ABSTRACT

DU, XUELIAN. Characterization of the dinuclear metal center of *Pyrococcus furiosus* prolidase and production of its mutants with increased activity at low temperature. (Under the direction of Dr. Amy M. Grunden).

Prolidase isolated from the hyperthermophilic archaeon *Pyrococcus furiosus* has potential for application under harsh conditions for decontamination and detoxification of organophosphorus compounds contained in certain pesticides and chemical warfare agents. However, this application is greatly restricted by two major factors.

The first factor comes from current knowledge of this enzyme. Previous study suggested *P. furiosus* prolidase contained two cobalt atoms with different affinity at the catalytic center which are required for full activation. However, no data had established which Co site was tight-binding and which was loose-binding. To clearly address this question, we used site-directed mutagenesis to modify amino acid residues that participate in binding the Co1 site (E313 and H284), the Co2 site (D209) or bidentate ligand site (E327). Metal-content, enzyme activity and CD-spectra analyses of D209A-, H284L- and E327L-prolidase mutants show that *P. furiosus* prolidase contains a dinuclear metal center with Co1 serving as the tight-binding and Co2 the loose-binding sites. Results of this study not only provides insight into the nature of *P. furiosus* prolidase active center, but facilitates our understanding of the mechanisms involved in enzyme catalysis.

The second factor that limits the application of *P. furiosus* prolidase is its narrow temperature range for catalytic activity. *P. furiosus* prolidase exhibits extreme high activity at 100°C but negligible activity at low temperatures. To improve the enzyme's catalytic activity at low temperatures, *P. furiosus* prolidases were randomly mutagenized and screened

at room temperature for increased activity. This study led to the identification of two low-temperature adapted prolidase mutants with one having Gly39 substituted by glutamate (G39E) and the other having Glu236 substituted by valine (E236V). G39E- and E236V-prolidases were further characterized to obtain better understanding of substrate catalysis at both low and high temperature and the relationship of these features with thermoactivity and thermostability.

Characterization of the dinuclear metal center of *Pyrococcus furiosus* prolidase and production of its mutants with increased activity at low temperatures

by

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DEDICATION

I dedicate this degree to my parents Yanqing Du and Caiyun Chen, my brother Zhiyong Du, and especially my husband Xiang Wang. Thank you for being there for me all these years and giving me strength and courage to overcome difficult moments. Without your support and understanding, I could not have come so far. I love you all.

BIOGRAPHY

Xuelian Du was born on January 18, 1977, in Wuhan, a city in central China with a population of over 7 million. Growing up with company of her lovely pets, which included dogs, cats, rabbits, chickens, ducks, parrots, gold fish, and turtles, she seemed destined to study biology in the future. In the fall of 1995, Xuelian attended Hubei University in China, and four years after she obtained a B. S degree in Biotechnology. During that time, she worked in the Molecular biochemistry lab led by Dr. Lixin Ma, where she conducted the expression of oligo-1,6-glucosidase from *Bacillus subtilis* in *Pichia pastoris*. After a short stint as a supply-chain manager at Canon China in 2000, Xuelian realized science is more fun than business, and soon she made a big decision to go abroad with her husband to continue her studies in Microbiology. In 2002, Xuelian enrolled in the Ph. D. program in the Department of Microbiology at North Carolina State University, where she started her research on thermostable Achaea under the guidance of Professor Amy Grunden.

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I have been lucky to work with my lab colleagues and friends Alice Lee, Mikyoung Ji, Drew Devine, Casey Theriot and Oscar Tirado-Acevedo. They have helped me understand and get involved in American culture, have witnessed the moments that I struggled on my projects and oftentimes have helped me out by listening to me or providing helpful suggestions. I have always enjoyed working with them and I will always cherish our friendship.

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CHAPTER 1

Literature Review

1.1 The hyperthermophilic archaeon *Pyrococcus furiosus*

Hyperthermophiles are organisms that reproduce and grow at very high temperatures and have optimum growth temperatures between 80°C and 110°C [1]. They were first isolated in the early 1980s through the pioneering efforts of Stetter and coworkers [2]. To date, more than 70 hyperthermophiles have been described with the majority classified as Archaea and the rest as Bacteria [1].

As a consequence of the growth temperatures of hyperthermophiles, enzymes isolated from these organsims are typically active and stable at high temperatures. This feature provides hyperthermophilic enzymes the following major biotechnological advantages over mesophilic enzymes: 1) once expressed in mesophilic hosts, hyperthermophilic enzymes are easy to purify by heat treatment, 2) their thermostability is associated with a higher resistance to chemical denaturants (such as solvent or guanidinium hydrochloride), and 3) performing enzymatic reactions at high temperatures allows higher substrate concentrations, lower viscosity, a decreased risk of microbial contamination and often higher reaction rates [1].

To date, enzymes isolated from hyperthermophiles have been used in areas including medicine, food production and as research reagents, and a major commercial application of a thermostable enzyme so far is the polymerase-chain-reaction (PCR) employing the DNA polymerase of the hyperthermophile *Pyrococcus furiosus* [3]. In addition to the direct application of these thermostable enzymes in technical processes, it is also fundamentally important to understand the thermoprotection mechanisms that enable hyperthermophiles to grow at high temperature, and to characterize the basic biochemistry and physiology of hyperthermophiles and how they differ from mesophilic

prokaryotes. The success of such endeavors could enable the rational design of important mesophilic enzymes where high thermostability is required.

Among these hyperthermophiles, *Pyrococcus furiosus* is the most studied hyperthermophilic archaea at present. *P. furiosus* is a strict anaerobe usually found in shallow or deep-sea hydrothermal vent systems [3]. It has an optimum growth temperature between 96°C and 100°C, and the maximum growth temperature of 105°C. The pH range for its growth is from 5 to 9 [3]. Like many heterotrophic organisms, *P. furiosus* utilizes protein, peptides and sometimes simple sugars as carbon and energy sources and produces organic acids, CO_2 and H_2. This organism was first isolated by Stetter and Fiala near Vulcano Island, Italy in 1986 [4]. Since then, thousands of papers have been published about *P. furiosus*, many of them focused on understanding this organism's metabolism and the adaptations it uses to survive in its extreme environment.

The pathways of carbohydrate and peptide metabolism in *P. furiosus* have been well studied [5, 6]. Specifically, glycolysis appears to occur via a modified Embden-Meyerhof pathway (Figure 1-1) [7]. This pathway is unusual in that the hexose kinase and phosphofructokinase steps are dependent on ADP rather than ATP, and in addition, a novel tungsten-containing enzyme termed glyceraldehyde-3-phosphate: ferredoxin oxidoreductase (GAPOR) replaces the expected glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase. Amino acid catabolism in *P. furiosus* is thought to involve four distinct 2-keto acid oxidoreductases that convert transaminated amino acids into their corresponding coenzyme A (CoA) derivatives (Figure 1-2) [8-11]. These CoA derivatives, together with acetyl-CoA produced from glycolysis via pyruvate, are then transformed to their corresponding organic acids by two

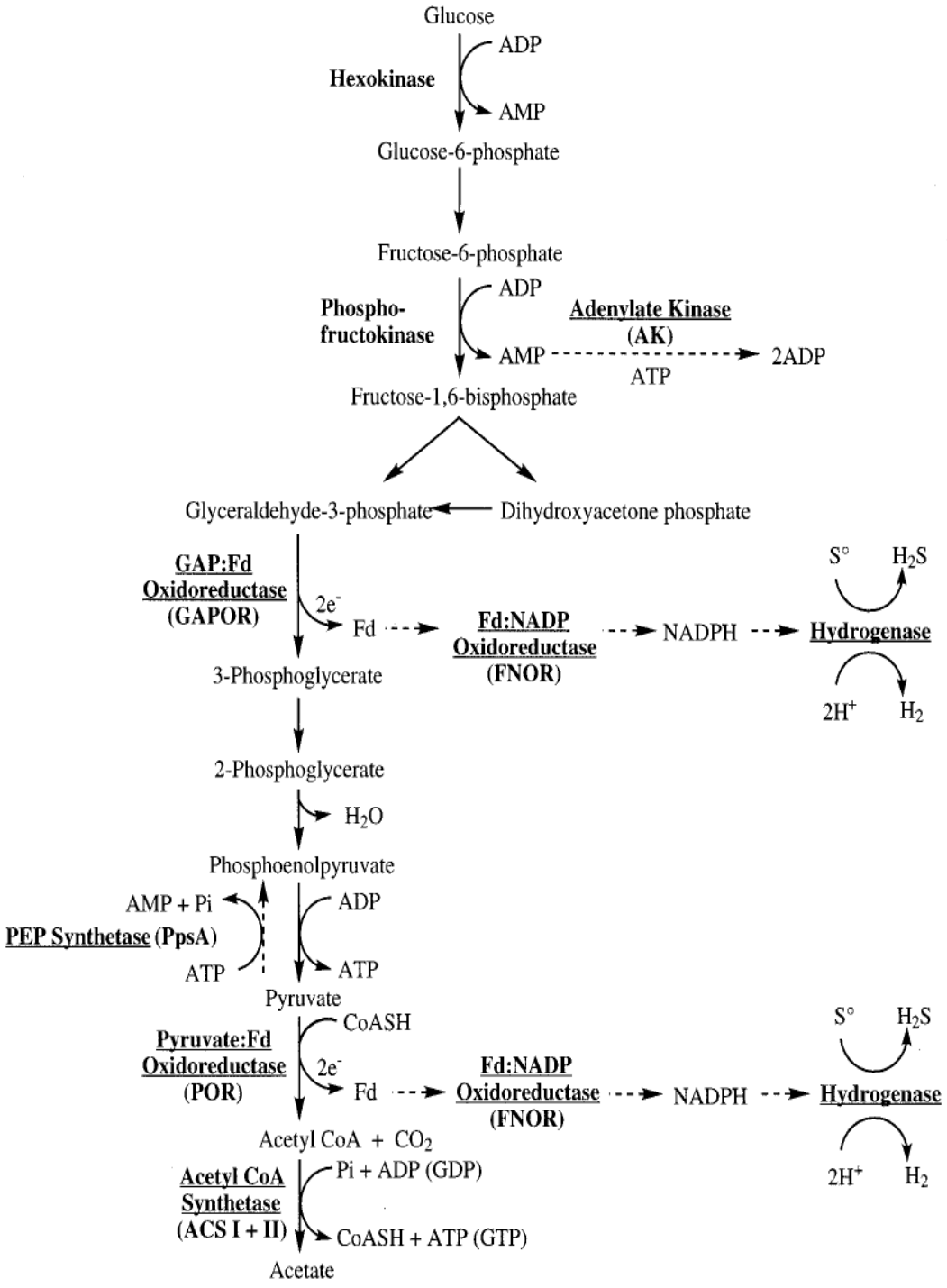

Figure 1-1. Proposed glycolytic pathway in *P. furiosus* [12].

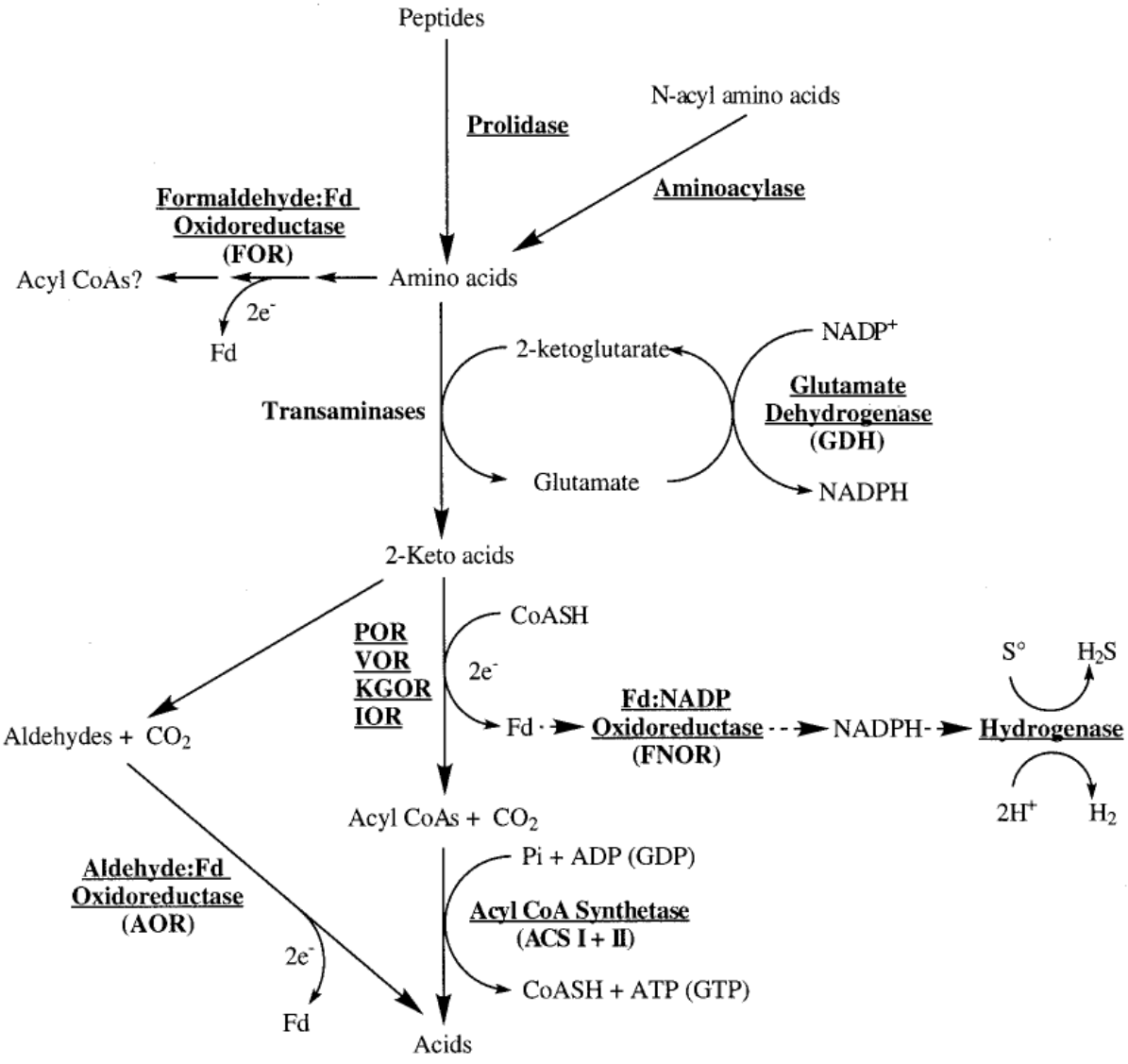

Figure 1-2. Proposed peptidolytic pathway in *P. furiosus* [12].

acetyl-CoA synthetases, unique to archaea, with concomitant substrate-level phosphorylation to form ATP [13]. Alternatively, depending on the redox balance of the cell, 2-keto acids could be decarboxylated to aldehydes and then oxidized to form carboxylic acids by a second tungsten-containing enzyme, aldehyde:ferredoxin oxidoreductase (AOR) [14, 15]. A third enzyme of this type, termed formaldehyde:ferredoxin oxidoreductase (FOR), is thought to be involved in the catabolism of basic amino acids [16]. Despite continuing studies on the carbohydrate and peptide metabolism pathways of *P. furiosus*, a good understanding of proteases and peptidases in the organism remains unclear.

Proteases and peptidases are critical to the maintenance of cellular function of *P. furiosus*. Because of the extremely low organic matter content of the submarine environment, proteases are required to help *P. furiosus* obtain its energy and carbon from complex mixtures of peptides scavenged from primary producers [1]. The proteases also recognize and break down unneeded or abnormal polypeptides in the cells, which can be produced as a result of environmental stress, mutation or errors in biosynthetic processes [17]. Initial efforts to study *P. furiosus* indicated that the organism was highly proteolytic [18-20]. A further analysis of the *P. furiosus* genome emphasized this point, revealing the presence of about 40 genes encoding proteases, protease subunits, or peptidases [21]. Despite their important physiological roles, only a few proteases and peptidases so far have been isolated and characterized from *P. furiosus*. These are a membrane-associated serine protease, pyrolysin [22], an intracellular protease with trypsin- and chymotrypsin-like activities [15], an intracellular endopeptidase that cleaves at prolyl residues [23], and prolidase, an intracellular dipeptidase that cleaves dipeptides with proline at the C-

terminus [24]. My research presented here is focused on the study of *P. furiosus* prolidase.

1.2 Physiological role of prolidase in *P. furiosus*

Prolidase is a proline hydrolase. Based on its enzymatic functions, prolidase is believed to participate, in concert with other endo- and exopeptidases, in the terminal degradation of intracellular proteins and may also function in the recycling of proline. It has substrate specificity for dipeptides with proline at the C-terminus (NH2-X-/-Pro-COOH), and cleaves on the N-terminal side of the proline.

Proline peptide bonds are very difficult to hydrolyze. In nature, only a few peptidases are known that are able to hydrolyze bonds adjacent to proline and these peptidases cover practically all situations where a proline residue might occur in a potential substrate. There are (i) proline-specific endopeptidases, which hydrolyze peptides on the carboxyl side of prolyl residues located internally within a polypeptide (-X-Pro-/-X); (ii) prolyl aminopeptidases, which cleave the bond between any N-terminal amino acid and a penultimate prolyl residue (NH2-X-/-Pro--X-) in peptides of various lengths; (iii) proline iminopeptidases, which catalyze cleavage of unsubstituted N-terminal prolyl residues from dipeptides, tripeptides, and polypeptides (Pro-/-X-); (iv) proline specific C-terminal exopeptidases (-X-Pro-/-X-COOH), which release an amino acid from the C terminus of a peptide with a penultimate proline residue; and (v) prolidases, which only cleave dipeptides with proline at the C terminus (NH2-X-/-Pro-COOH) (Figure 1-3) [25].

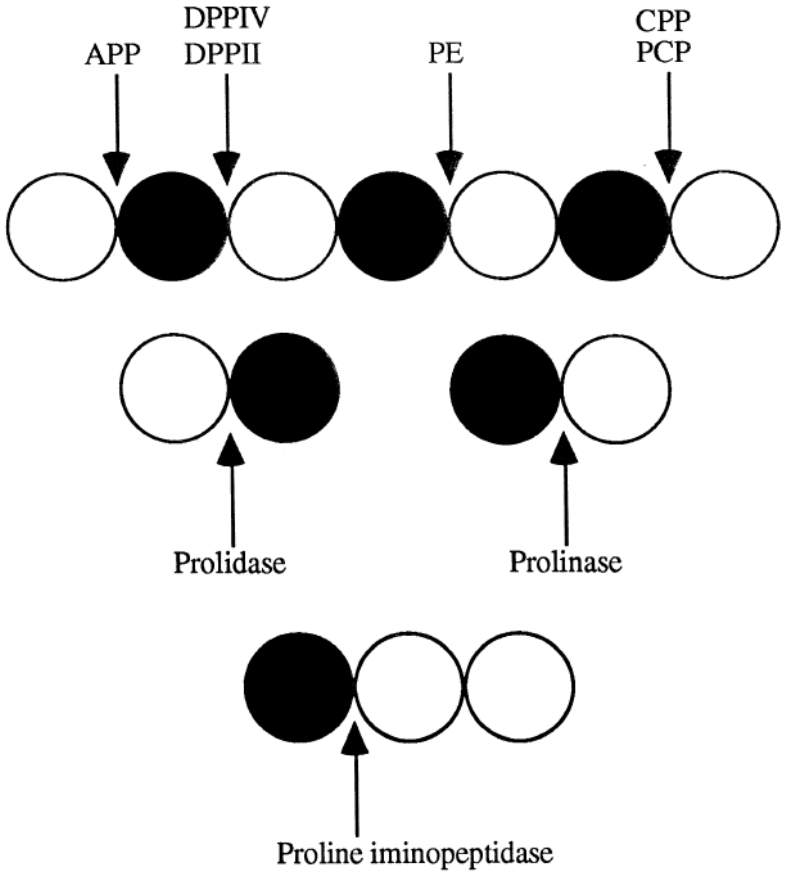

Figure 1-3. Versatility of proline-specific peptidases. This figure illustrates that for practically all situations where proline (•) may occur in a peptide or protein (ooo), there has evolved an enzyme capable of specifically recognizing this amino acid. APP, aminopeptidase P; DPPIV, dipeptidyl peptidase IV; DPPII, dipeptidyl peptidase II; PE, prolyl endopeptidase; CPP, carboxypeptidase P, PCP, prolyl carboxypeptidase [25].

The difficulty in hydrolyzing proline-containing peptide bonds is due to the unique conformational and biochemical attributes imparted by the presence of proline. Proline differs from other amino acids in that its side chain R group is bonded to both the amino group and the α–carbon, resulting in a cyclic structure (shown in Figure 1-4). Due to its unique structural properties, important conformational attributes are observed when proline is introduced into a peptide sequence. Its cyclic structure limits the angle of rotation about the α-carbon and nitrogen within a peptide bond. Consequently, proline introduces a fixed bend into the peptide chain which is a potent repeated structure breaker that tends to change the direction of peptide chains, a causative factor in the spherical or globular shape of proteins [26]. Moreover, proline plays a key physiological role in the protection of biologically active peptides against enzymatic degradation. Many peptides or protein precursors, such as hormonal peptides and neurotransmitters, are highly regulated by ordered series of post-translational modification [25]. Their active forms require the involvement of endopeptidases, which cleave its precursor at a specific site, followed by proteolytic ‘‘trimming’’ by exopeptidases to reduce the polypeptide chain to its correct size [25]. The peptide chain must possess some structural or biochemical element to prevent excessive hydrolysis with subsequent loss of biological activity. Proline residues situated within these polypeptide precursors act as structural elements limiting the susceptibility of the polypeptide chain to proteolysis and are present at the modification site prior to enzymatic processing of the precursor [25].

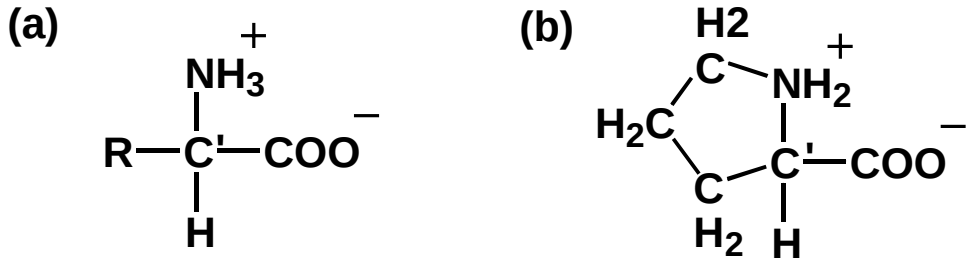

Figure 1-4. Structural characteristics of amino acids and proline. (a) General amino acid structure illustrating the carboxyl, amino, and R groups attached to a chiral α-carbon (C'). (b) Proline, where the bonded amino group and R group $–CH_2–CH_2–CH_3$ create a cyclic structure. The secondary nature of the nitrogen containing moiety makes proline an imino acid [25].

1.3 Characterization of *P. furiosus* prolidase

The prolidase from the hyperthermophilic archaeon *Pyrococcus furiosus* has been isolated and characterized by Ghosh and Grunden [24]. They also obtained the recombinant form of the enzyme from expression in *Escherichia coli* [24]. The enzyme is a homodimer with a subunit molecular mass of 39.4 kDa. When purified from either *P. furiosus* or *E. coli*, *P. furiosus* prolidase contains only one Co (II) atom per subunit. Full catalytic activity requires the addition of Co^{2+} ions, indicating that the enzyme has a second metal binding site. Both native and recombinant *P. furiosus* prolidases are very thermostable, with no loss in the activity for 12 h for native *P. furiosus* prolidase and 50% loss in the activity ($t_{50\%}$) for 6 h for recombinant *P. furiosus* prolidase when 0.3 mg/ml samples was incubated in a sealed vial at 100°C. In the assay, Co^{2+} can be replaced by Mn^{2+}, but not by Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, or Ni^{2+}. A concentration of 1.2 mM $CoCl_2$ provided *P. furiosus* prolidase maximal activity, whereas above this concentration, this cation caused some inhibition. *P. furiosus* prolidase has a narrow substrate specificity, hydrolyzing only dipeptides Xaa-Pro, where Xaa is nonpolar (Met, Leu, Val, Phe, Ala). The optimal activity with Met-Pro as the substrate occurs at pH 7.0 and 100°C.

Kinetic and metal analyses indicated that *P. furiosus* prolidase (both native and recombinant versions) has at least two binding sites per subunit for Co^{2+} ions. One appears to be an integral part of the protein and not removed by purification or dialysis, while the other has an association constant of ≈0.3 mM and is essential for catalysis. In this regard, the *P. furiosus* enzyme resembles certain members of the broad class of binuclear metallohydrolases represented by *E.coli* methionine aminopeptidase (MAP)

[27], *E. coli* proline aminopeptidase (APPro) [28], bovine lens leucine aminopeptidase (bLeuAP) [29], *Aeromonas proteolytica* aminopeptidase (ApAP) [30], *Streptomyces griseus* aminopeptidase (SgAP) [31], human methionine aminopeptidase-2 (HsMetAP) [32], *Pyrococcus furiosus* methionine aminopeptidase-2 (PfMetAP) [33], and carboxypeptidase G2 from *Pseudomonas* sp. strain RS-16 [34]. What these peptidases all have in common is that their active sites also contain two metal ions that typically differ in their exchange kinetics. However, *P. furiosus* prolidase is structurally closer to *E. coli* MAP in that they are the only two that are preferentially cobalt-dependent.

Further alignment of the amino acid sequence of *P. furiosus* prolidase with *E. coli* methionine aminopeptidase (MetAP) revealed that the five amino residues (Asp97, Asp108, His171, Glu204 and Glu235) acting as metal ligands in *E. coli* MAP was also conserved in *P. furiosus* prolidase (Asp209, Asp 220, His280, Glu313 and Glu327), indicating *P. furiosus* prolidase, like MAP, may also contain a dinuclear metal center in the active site of each subunit, with His280 and Glu313 solely binding to the first Co (Co_1), Asp209 to the second Co (Co_2), and Asp220 and Glu327 liganding both cobalt atoms (Figure 1-5).

Using X-ray crystallographic analysis of *P. furiosus* prolidase, Maher and Ghosh confirmed this proposed dinuclear metal center structure [35]. They determined that *P. furiosus* prolidase exists as a homodimer, with each subunit having an N-terminal domain (domain I, residues 1-112), a α-helical linker (residues 113-123), and a C-terminal domain (domain II, residues 124-348) [35]. Domain I consists of a six-stranded mixed β-sheet flanked by five α-helices (three on one side and two on the other). Domain II is formed around a mixed six-stranded β-sheet with four α-helices on the outer surface. The

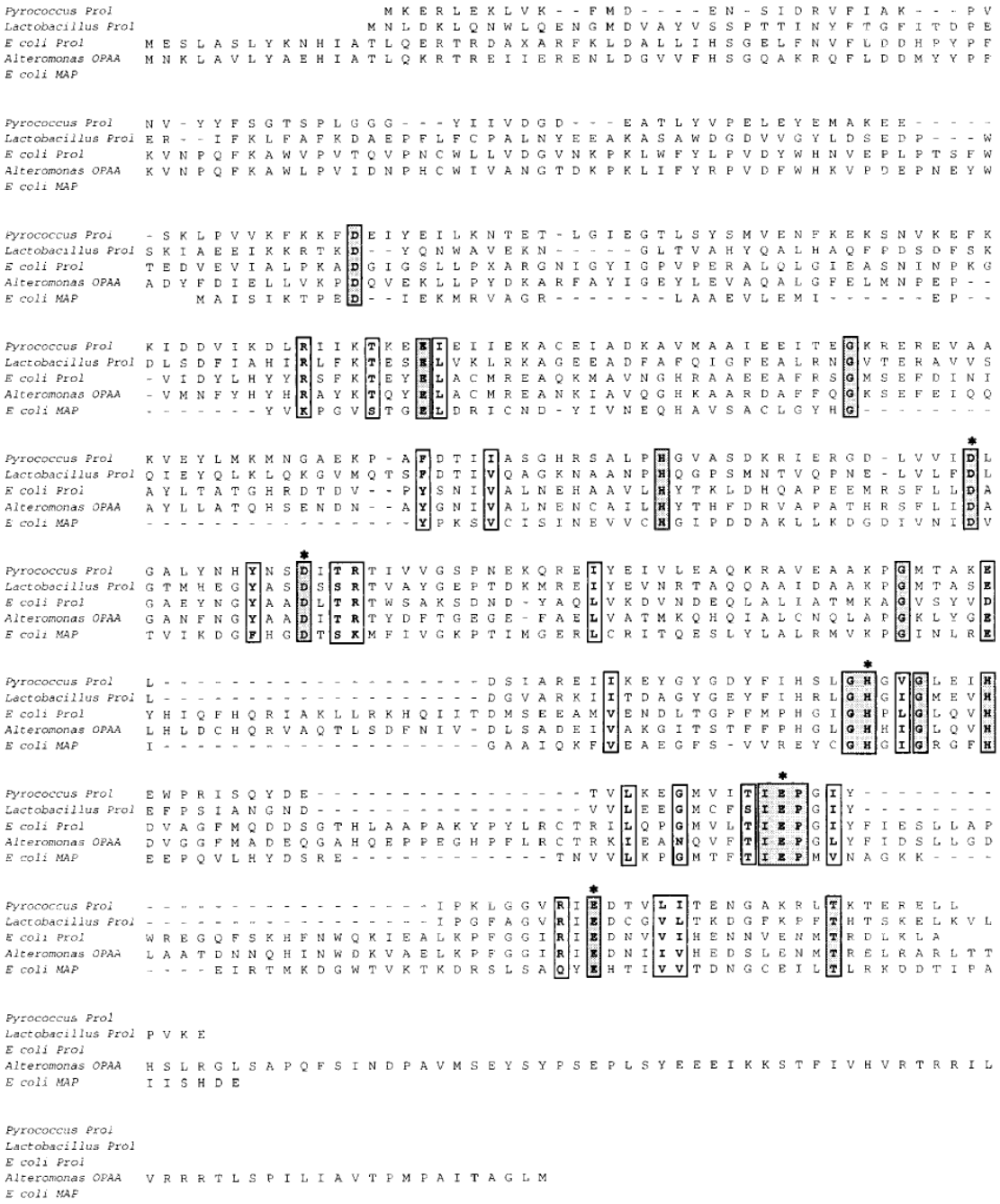

Figure 1-5. Alignment of the amino acid sequence of *P. furiosus* prolidase with other prolidases (Prol), *Alteromonas* OPAA, and *E. coli* methionine aminopeptidase (MAP). The five residues that are ligands to the binuclear cobalt site in the subunit of *E. coli* methionine aminopeptidase are indicated by asterisks [24].

β–sheet is strongly curved to form a typical “pita-bread” fold [35] and the enzymatic active center is within the “pita-bread” fold with its central feature as a dinuclear metal cluster. The metal atoms are coordinated by the side chains of two aspartate residues (Asp209 and Asp220), two glutamate residues (Glu313 and Glu327), a histidine (His284), and a bridging atom modeled as water (W176). The carboxylate groups of Asp220 and Glu327 act as bridges between the metal atoms (Figure 1-6) [35].

In 1994, it was suggested by Bazan and Weaver that methionine aminopeptidase, prolidase, aminopeptidase P and creatinase shared a common fold from their sequence similarities [36]. This suggestion has been supported by Maher and Ghosh [35] by searching the Protein Data Bank using the *P. furiosus* prolidase coordinates, which indicated that the proteins whose structures are most closely related to that of *P. furiosus* prolidase are APPro from *E. coli*, the MAPs from *E. coli* and creatinase from *Pseudomonas putida* [35]. For this reason, prolidase, MAP, APPro and creatinase have been classified into the same subclass of metallopeptidases in a review paper by Lowther and Matthews [37]. Their most distinctive feature is a pita-bread fold of the C-terminal catalytic domain with a dinulcear metal center.

1.4 Proposed reaction mechanism

Biochemical and structural analyses of MAP, APPro and prolidase suggest they have a similar catalytic mechanism [37]. Therefore, some well-established catalytic mechanisms of E.coli MAP can serve as good a comparison for the consideration of the possible reaction mechanism of prolidase. Specifically, mutation of active-site residues, together with the structures of complexes of *E.coli* MAP with substrate-like inhibitors,

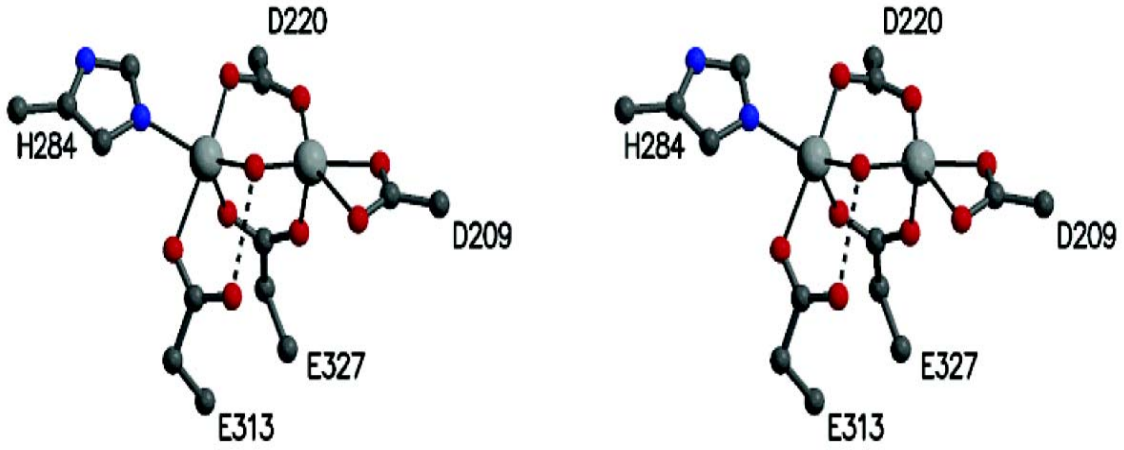

Figure 1-6. Part of X-ray diffraction structure showing the active site of *P. furiosus* prolidase. The metal atoms are shown as gray spheres. A hydrogen bond between Glu313 and the bridging hydroxide ion is shown as a dotted line [35].

phosphorus-based transition-state analogs and reaction products have provided significant insights into the reaction mechanism of prolidase [37, 38].

It is proposed that, for *E. coli* MAP, the cleavage of peptide bond occurs as follows: the substrate binds with the N-terminus interacting with three potential acidic residues in a manner that remains to be determined (intermediate I). The carbonyl and amide groups of the scissile peptide bond interact with M1 plus His178 and His79, respectively. M1 and Glu204 of *E. coli* MetAP facilitate the activation of a water molecule to become a nucleophilic hydroxide ion. Two interactions to M1 and one to His178 stabilize the tetrahedral intermediate (intermediate II). Breakdown of the intermediate is facilitated by donation of a proton from Glu204 to the amine of the leaving group. The resulting product complex (intermediate III) maintains interactions with M1 and His178 (shown in Fig. 1-7) [37].

Given the biochemical and structural similarity between *E. coli* MAP and *P. furiosus* prolidase, it is also reasonable to apply this model to *P. furiosus* prolidase, with three amino residues His79, His178 and Glu204 in *E. coli* MAP corresponding to His192, His291 and Glu 313 in *P. furiosus* prolidase.

1.5 General features of prolidases from other organisms

Prolidase is widespread in nature and has been isolated and characterized from different sources other than *P. furiosus*. Prolidase was first discovered during investigations into aminopeptidase and carboxypeptidase activities in porcine intestinal mucosal extracts [39] . Subsequently, it has been purified and characterized from many different mammalian tissues, such as calf brain [40], swine kidney [41], human

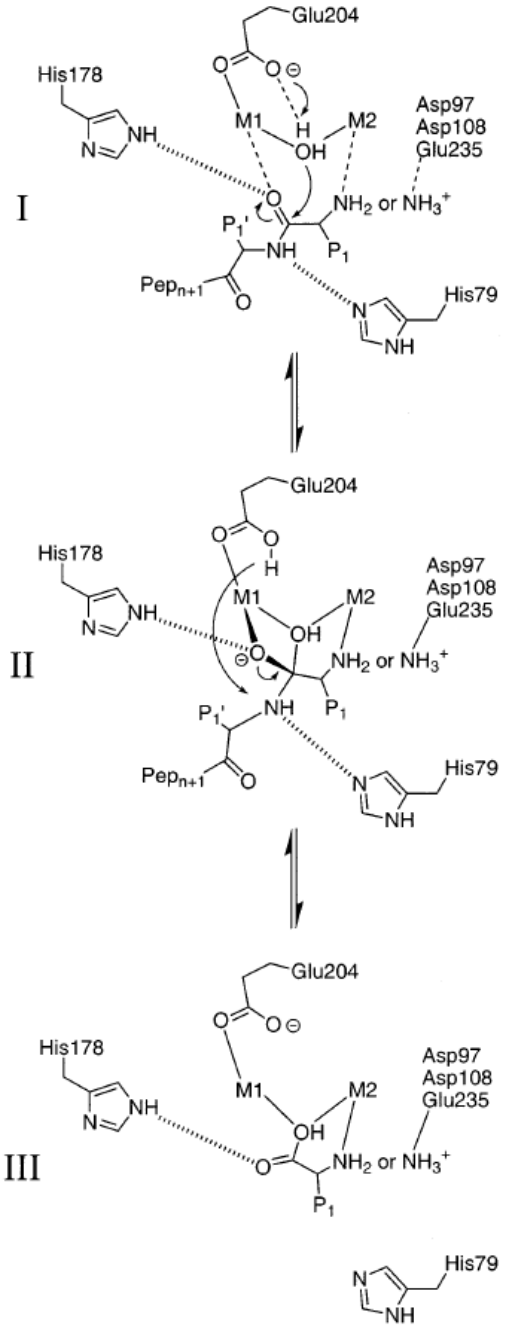

Figure 1-7. Proposed reaction mechanism for the hydrolysis of peptides by *E. coli* methionine aminopeptidase [37].

erythrocyte [42], and pig and monkey intestine [43-45]. It is has also been isolated from a number of bacteria (*Lactobacillus* sp., *Xanthomonas maltophilia*, *Alteromonas* sp., *Streptococcus cremoris*, *Aureobacterium esteraromaticum*, *Aspergillus nidulans*) and one archaeon (*Pyrococcus furiosus*) [24, 46-53]. Based on its enzymatic functions, prolidase is believed to participate, in concert with other endo- and exopeptidases, in the terminal degradation of intracellular proteins and may also participate in the recycling of proline. In humans, prolidase is responsible for mediating the final step of collagen degradation and recycling. Prolidase deficiency in humans also causes C-terminal proline-containing dipeptides to accumulate to toxic concentrations in individuals. Prolidase deficiency in humans ultimately leads to a disease characterized by various skin manifestations accompanied by mental retardation, facial dysmorphism and susceptibility to pyogenic infections [26, 54, 55].

Biochemical characterization of prolidases from different sources indicate that most of the prolidases are metal dependent [37]. The enzyme activity is completely inhibited by strong metal-coordinating agents such as EDTA or 1,10- phenanthroline, whereas the addition of the divalent metals restores enzyme activity [37]. Maximal prolidase activity requires divalent cations. However, this metal requirement varies for prolidases from different sources. For instance, prolidases isolated from human erythrocytes, *Xanthomonas maltophilia. L. casei* and *Aspergillus nidulans* require Mn^{2+} for maximal activity [42, 47, 50, 53] whereas prolidase isolated from *Pyrococcus furiosus* requires Co^{2+} for maximal activity [24].

Besides metal variability, the number of subunits present in the active forms of prolidase from different sources varies. For example, prolidase isolated from mammalian

tissues (guinea pig brain, human erythrocytes) and the archaeon *P. furiosus* are catalytically active as dimers (subunits are from 39 to 58 kDa) [24, 56, 57], whereas the enzymes from *Lactobacillus lactis* and *L. casei* are monomers (42kDa) [50, 58].

It is also very intriguing that, among all the prolidases characterized so far, only *P. furiosus* prolidase is highly selective in hydrolyzing dipeptides with proline at the C-, but not the N-terminus of the dipeptide [24]. The prolidases of *L. lactis* and *A. esteraromaticum* hydrolyze dipeptides with Pro at either the N- or C-terminal position [51, 58], and the enzymes from *L. casei* and guinea pig brain efficiently cleave some dipeptides with no prolyl residue [50, 56]. These differences in substrate specificities among the characterized prolidases are likely determined by the different number of subunits present in the active enzyme as well as the nature of the metal ion occupying the active site [59].

1.6 Potential industrial applications of prolidase

Prolidase is believed to participate in the degradation of intracellular proteins and in proline recycling. In addition to the physiological role it plays in nature, the prolidase enzyme also has potential biotechnological/industrial applications. For example, the enzyme is used in the dairy industry as a cheese-ripening agent, since release of proline from casein by prolidase will reduce bitterness. Casein is the preliminary protein in milk and is very rich in proline: e.g. 17 of the 199 residues in α_{s1}-casein and 35 of the 209 residues in β-casein are proline [60]. It has been reported that dipeptides containing proline have a bitter taste [61], whereas free proline imparts a sweet flavor to cheese [62]. An analysis of proteinase activity from *Lactococcus* strains further proved that the strain

that exhibited higher prolidase activity produced a better flavored cheese product, indicating this enzyme may contribute to the cheese flavor development [60].

Another potential application of prolidase came from the finding that an organophosphorus acid anhydrolase (OPAA) from *Alteromonas* sp., which was later determined to be a prolidase, can hydrolyze organophosphorus (OP) compounds. OPs are present in certain pesticides and chemical warfare agents, which, in some cases, are highly toxic and hard to degrade [48, 63]. Examples of these OP compounds include: the serine protease inhibitor diisopropyl fluorophosphates (DFP), and different G-type nerve agents such as soman (GD; *O*-pinacolyl methylphosphonofluoridate), sarin (GB; *O*-isopropyl methylphosphonofluoridate), and GF (*O*-cyclohexyl methylphosphonofluoridate) [64]. Exposure to these compounds will irreversibly inhibit peripheral and central acetylcholinesterase (AChE), a key enzyme responsible for terminating the action of the neurotransmitter acetylcholine [65]. An inhibition of this enzyme results in a build up of acetylcholine and causes an over stimulation of muscarinic and nicotinic receptors. An accumulation of acetylcholine results in nerve agent poisoning, which is characterized by hypersecretion, convulsions, respiratory distress, coma and death [65].

Current decontamination solutions to remove those toxic OP compounds include the cleaning solution DS2 and bleach [66]. Despite high efficiency, both DS2 and bleach are very corrosive to the treated surface and also result in hazardous wastes [66]. The finding that prolidase can hydrolyze those OP compounds by cleaving P-F, P-O, P-CN and P-S bonds provides a potential alternative way to decontaminate and detoxify the OPs on an exposed surface without introducing new hazardous waste.

1.7 The advantages and limitations of *P. furiosus* prolidase for use in decontamination of toxic organophosphorus nerve agents

Among all prolidases that have been characterized so far, the ones that have been isolated from mesophilic sources are maximally active only at temperatures up to 55°C. However, the prolidase that was isolated from the hyperthermophilic archaeon *Pyrococcus furiosus* has a maximum activity at 100°C. In addition, both native *P. furiosus* prolidase and recombinant *P. furiosus* prolidase produced in *E. coli* possess significant thermostability, with the former displaying no activity loss after 12 hours incubation at 100°C and the latter exhibiting 50% activity loss after 6 hours incubation at 100°C [24]. This high degree of thermostability makes *P. furiosus* prolidase ideal for some biotechnological applications where harsh conditions, such as high temperatures, organic solvents and denaturants, are required [67].

Despite the significant potential of *P. furiosus* prolidase in industrial applications, several problems need to be addressed before its unique properties can be fully harnessed. An important problem came from the fact that despite numerous studies done to characterize prolidase structures, there remained questions in regards to the exact nature of the *P. furiosus* prolidase metal center, which plays a very critical role in the enzyme catalysis. It is known that the *P. furiosus* prolidase metal center binds two cobalt atoms with different binding affinity [24], however no existing structural data had shown which cobalt is tightly bound and which cobalt is loosely bound. Furthermore, the recent findings that the highly related enzymes *E. coli* MAP and *P. furiosus* MAP are likely Fe-containing mononuclear proteases rather than dinuclear proteases as first thought [68, 69], provided reason to evaluate this possibility for *P. furiosus* prolidase as well. To

answer these questions, our first project was designed to characterize the relative affinities of the metal atoms in the dinuclear metal centers in *P. furiosus* prolidase by analysis of targeted *P. furiosus* prolidase mutants. The specific amino acid residues that were shown to participate in binding the metal centers based on X-ray crystal structure analysis were targeted for mutation. The purified *P. furiosus* prolidase mutants (D209A-, H284A-, H284L- and E327L-prolidase) were evaluated using metal content, circular dichorism (CD) spectra and activity analyses in an effort to establish the identity of the tight-binding and loose-binding Co centers in *P. furiosus* prolidase. A clearer understanding of the nature of the metal centers in *P. furiosus* prolidase will enable a structure-informed design for engineering prolidases better suited for toxic OP nerve agent decontamination.

Another factor limiting the utility of *P. furiosus* prolidase for OP nerve agent decontamination is the temperature range in which the enzyme is catalytically active. *P. furiosus* prolidase has optimal activity at 100°C and displays little activity at temperatures below 50°C and has only 50% activity at 80°C [24]. Therefore, it is desirable to attempt to prepare and screen *P. furiosus* prolidase mutants that demonstrate increased activity against OP compounds at lower temperature (25°C to 50°C) while retaining its thermostability. Besides the possibility of obtaining a better enzyme for OP compound decontamination, isolation of such *P. furiosus* prolidase mutants would also afford the important opportunity to investigate structural factors that influence protein thermoactivity, an area of study that remains underdeveloped. For this reason, my second project was designed to produce *P. furiosus* prolidase mutants with increased catalytic activity at lower temperature.

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CHAPTER 2

Characterization of the dinuclear metal center of *Pyrococcus furiosus* prolidase by analysis of targeted mutants

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ABSTRACT

Prolidases are dipeptidases specific for cleavage of Xaa-Pro dipeptides. *Pyrococcus furiosus* prolidase is a homodimer having one Co-bound dinuclear metal cluster per monomer with one tightly bound Co (II) site and the other loosely bound (K_d 0.24 mM). To identify which Co site is tight-binding and which is loose-binding, site-directed mutagenesis was used to modify amino acid residues that participate in binding the Co1 (E-313 and H-284), the Co2 site (D-209) or the bidentate ligand (E-327). Metal-content, enzyme activity and CD-spectra analyses of D209A-, H284L, and E327L-prolidase mutants show that Co1 is the tight-binding and Co2 the loose-binding metal center.

2.1 INTRODUCTION

Prolidase is a proline hydrolase that specifically cleaves dipeptides with proline at the C terminus (NH2- X-/-Pro-COOH) [1]. It is widespread in nature and has been isolated from a variety of mammalian tissues [2-5], bacterial and archaeal sources [6-10]. Prolidases in bacteria and archaea are thought to participate, in concert with other endo- and exopeptidases, in the degradation of intracellular proteins and in proline recycling based on their enzymatic function. In humans, prolidase is involved in the final stage of the degradation of endogenous and dietary protein, and is particularly important in collagen catabolism. Mutations in the gene encoding for human prolidase cause prolidase deficiency, an autosomal recessive disorder characterized by skin lesions, mental retardation and recurrent infections [11-14].

To date, the majority of prolidases that have been studied exhibit metal-dependent activity, requiring divalent cations such as Zn^{2+}, Mn^{2+}, or Co^{2+} for maximal activity [15, 16]. Purified prolidases have been shown to exist either as monomers or dimers depending on the source [1, 17]. Although all characterized prolidases preferentially hydrolyze Xaa-Pro dipeptides, some prolidases can cleave dipeptides with proline as the N-terminal residue or can hydrolyze dipeptides that do not contain a prolyl residue [3, 6, 7, 10]. These differences in substrate specificities among the characterized prolidases are likely a function of differences in the numbers of subunits present in the active enzyme as well as the nature of the metal ion occupying the active site.

In addition to its function as a peptidase, prolidase has several biotechnological applications. The enzyme is used in the dairy industry as a cheese-ripening agent, since

the release of proline from peptides in cheese reduces bitterness [18]. It has also been found that an organophosphorus acid anhydrolase (OPAA) from *Alteromonas* sp., which was later determined to be a prolidase, can hydrolyze organophosphorus (OP) inhibitors present in certain pesticides and chemical warfare agents [19-21]. It is thought that this ability of prolidase may arise from the fortuitous similarity of these compounds in shape, size and surface charge to the true prolidase X-Pro dipeptide substrate.

Among all prolidases that have been characterized so far, the ones that have been isolated from mesophilic sources are only maximally active at temperatures up to 55 ºC. However, the prolidase that was isolated from a hyperthermophilic archaeon *Pyrococcus furiosus* has a maximum activity at 100 ºC. Both native and recombinant (produced in *Escherichia coli* using a T7 RNA polymerase-driven expression plasmid) forms of *P. furiosus* prolidase have been purified and biochemically characterized [9, 22]. The native and recombinant form of *P. furiosus* prolidases exhibit essentially equivalent physical and catalytic properties, and therefore, the recombinant version of the protein was used for the study presented here.

P. furiosus prolidase has narrow substrate specificity, hydrolyzing only dipeptides with proline at the C-terminus and a nonpolar amino acid (Met, Leu, Val, Phe or Ala) in the N-terminal position. Optimal activity was observed at pH 7.0 and a temperature of 100 ºC [9]. *P. furiosus* prolidase is active as a homodimer (39.4 kDa per subunit) and contains one Co^{2+} per subunit as purified [9]. Its catalytic activity requires the addition of Co^{2+} to the assay indicating that the enzyme has a second Co^{2+} binding site (K_d 0.24 mM) [9]. The enzyme activity could also be supported by the presence of Mn^{2+}, but not by the

addition of other divalent ions (Mg^{2+}, Ca^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+} or Zn^{2+}) under aerobic assay conditions [9].

X-ray crystal structure analysis of *P. furiosus* prolidase has identified five amino acids that function as the metal-binding residues in this enzyme. histidine-284 and glutamate-313 solely bind to the first Co center (Co1), aspartate-209 to the second Co center (Co2), and aspartate-220 and glutamate-327 ligand both cobalt atoms (shown in Figure. 2-1) [23, 24]. These five residues are also conserved in the dinuclear metal center of a variety of methionine aminopeptidases (MetAP) (type 2 human MetAP, *E. coli* MetAP and *P. furiosus* MetAP) and aminopeptidase P (APP) (*E. coli* proline aminopeptidase, bovine lens leucine aminopeptidase, *Streptomyces griseus* aminopeptidase) [16]. Based on their structural homologies and similar metal center properties, *P. furiosus* prolidase, MetAP and APP have been classified into the same subclass of metallopeptidases [16].

Despite the numerous studies done to characterize prolidase structures, there remain questions in regards to the exact nature of the metal centers in prolidases. For example, it is known that the *P. furiosus* prolidase metal center binds two cobalt atoms with different binding affinity [9], however no existing structural data has shown which cobalt is tightly bound and which cobalt is loosely bound. Furthermore, the recent findings that the highly related enzymes *E. coli* MetAP and *P. furiosus* MetAP are likely Fe-containing mononuclear proteases rather than dinuclear proteases as first thought, provides reason to evaluate this possibility for *P. furiosus* prolidase as well [25, 26].

To answer these questions, and to further characterize the relative affinities of the metal atoms in the dinuclear metal centers in *P. furiosus* prolidase, the specific amino

acid residues that were shown to participate in binding the metal centers based on X-ray crystal structure analysis [23, 24] have been targeted for mutation. The purified *P. furiosus* prolidase mutants (D209A-, H284A-, H284L- and E327L-prolidase) were evaluated using metal content, CD spectra and activity analyses in an effort to establish the identity of the tight-binding and loose-binding Co centers in *P. furiosus* prolidase. This clearer understanding of the nature of the metal centers in *P. furiosus* prolidase will lead to a better appreciation of prolidase function and could possibly lead to the development of prolidases optimized for OPAA nerve agent detoxification.

2.2 MATERIALS AND METHODS

2.2.1 Site-Directed mutagenesis of P. furiosus prolidase

The D209A-, E313L-, E327L-, H284A- and H284L-prolidase mutants were produced using the Quikchange site-directed Mutagenesis Kit (Stratagene) according to the supplier's recommendations. For the PCR mutagenesis, the wild type *P. furiosus* prolidase expression vector (pET-prol) was used as the DNA template, as were the following mutagenic primers: D209A-prolidase primer 1 (forward), GATTTAGTTGTTATTGCACTTGGAGCACTC and primer 2 (reverse), GAGTGCTCCAAGTGCAATAACAACTAAATC; E313L-prolidase primer 1 (forward), ATAGGATCCGGTGAGGAGGTTGTATGAAAGAAAGACTTGAA and primer 2 (reverse), ACCAAGCTTCGGAATGTATATGCCAGGAAGTATCGT; E327L-prolidase primer 1 (forward), ATTCCGAAGCTTGGTGGAGTTAGAATTCTTGACACG and primer 2 (reverse), ATAGGGATCCGGTGAGGAGGTTGTATGAAAGAAAGAC; H284A-prolidase primer 1 (forward), CTTCATCCACAGTCTAGGCGCAGGTGTTGGACTTGAGATTC and primer 2 (reverse), GAATCTCAAGTCCAACACCTGCGCCTAGACTGTGGATGAAG; and H284L-prolidase primer 1 (forward), ATCCACAGTCTAGGCCTTGGTGTTGGACTTGAG) and primer 2 (reverse), CTCAAGTCCAACACCAAGGCCTAGACTGTGGAT. The PCR was performed using a Bio-Rad thermocycler programmed for 16 cycles, with each cycle comprised of a denaturation step at 95 °C for 30 seconds, annealing step at 55 °C for 1 minute and extension step at 68 °C for 6 minutes. All of the mutant prolidase constructs were

sequenced over the entire length of the prolidase gene to confirm the presence of the target mutation and ensure that there were no other mutations.

2.2.2 Overxpression of the D209A-, E313L-, E327L-, H284A-, and H284L- prolidases

The mutant pET-prol plasmids that contained the D209A-prolidase, E327L-prolidase and H284A-, H284L-prolidase genes, respectively, were transformed into *E. coli* BL21(λDE3), which has isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression of T7-RNA polymerase. The transformants were grown as 1 L cultures in LB media incubated at 37 °C with shaking (200 RPM) until an optical density of 0.6-0.8 was reached. The expression of mutant prolidases was initiated when IPTG was added to the cell culture to a final concentration of 1 mM. The induced culture was incubated at 37 °C for 3 hours prior to the harvesting of the cells.

2.2.3 Purification of D209A-, H284A-, H284L-, and E327L-prolidase

Cell pellets containing D209A-, H284A-, H284L, and E327L-prolidase, respectively, were suspended in 50 mM Tris-HCl, pH 8.0 containing 1 mM benzamidine and 1 mM DTT (each 1 g {wet weight} of cell paste was suspended in 3 ml Tris-HCl buffer). The cell suspension was passed through a French pressure cell (20000 lb/in^2) twice. The lysed cell suspension was centrifuged at 20,000 RPM for 30 minutes to remove any cell debris and the supernatant was heat treated at 80 °C for 30 min anaerobically. The denatured protein was removed by centrifugation at 20,000 RPM for 30 minutes. The heat-treated extract of D209A-prolidase was applied to a DEAE column and the resulting D209A-prolidase fractions were subsequently applied to a Q column.

Fractions containing D209A-prolidase were then applied to a gel-filtration column. H284A-, H284L, and E327L-prolidases were also purified using the same steps as D209A-prolidase was except the DEAE column was replaced by a phenyl-sepharose column. $[NH4]_2SO_4$ was slowly added to the heat-treated extract with stirring to a final concentration of 1.5 M before the protein was loaded onto the phenyl-sepharose column. For the DEAE and Q column chromatography, the binding buffer used was 50 mM Tris-HCl, pH8.0 and proteins were eluted with a linear gradient of elution buffer: 50 mM Tris-HCl, 1 M NaCl, pH 8.0; for the phenyl-sepharose column chromatography, the binding buffer was 50 mM Tris-HCl, 1.5 M $(NH_4)_2SO_4$, pH 8.0 and the elution buffer was 50 mM Tris-HCl, pH 8.0. For the gel filtration column, the equilibration buffer was 50 mM Tris-HCl, 400 mM NaCl, pH8.0.

2.2.4 Enzyme assay

The enzyme activity assay used was based on a previously described method with slight modification indicated below [9]. Briefly, the assay mixture (500 µl) contained 50 mM MOPS buffer (3-[N-morpholino]propanesulfonic acid), pH 7.0, 4 mM Met-Pro (substrate), 5% (vol/vol) glycerol, 100 µg/ml BSA protein, 200 mM NaCl and 1.2 mM $CoCl_2$, and was incubated at 100 °C for 5 minutes. The reaction was initiated by addition of the enzyme. The mixture was incubated at 100 °C for 10 minutes, and the reaction was stopped by the addition of glacial acetic acid (500 µl) followed by the ninhydrin reagent (500 µl). Color development was achieved by heating the reaction mixture at 100 °C for 10 min. The solution was cooled to 23°C and the absorbance was determined at 515 nm. Specific activities were calculated using the extinction coefficient of 4,570 $M^{-1} \cdot cm^{-1}$ for

the ninhydrin-proline complex. For the assays performed to evaluate the activity of the wild type and mutant prolidases substituted with various metal ions (Co^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, or Zn^{2+}, the wild type and mutant prolidase enzymes were first treated with 5 mM EDTA to remove metal bound in the prolidase active sites using the method described below for the metal content analysis. The metal centers were subsequently reconstituted with 3-fold molar excess Co^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, or Zn^{2+} as indicated in the metal content analysis method, except that the reconstitution was conducted anaerobically. The reconstituted prolidase proteins were then assayed under either aerobic or anaerobic conditions.

2.2.5 Metal content analysis of the mutant P. furiosus prolidases

The prolidase samples that were to be analyzed for metal content were initially treated to remove any metal ions that may have been bound during the protein purification process. For this treatment, 5 ml of 1 mg/ml pure prolidase solution was injected into a slideAlyzer dialysis cassette (MWCO 10,000, Pierce Biotechnology) and dialyzed against 1 L 50 mM MOPS (pH 7.0) buffer containing 5 mM EDTA (repeated twice, 30 minutes each time). The dialyzed solution was then transferred to deionized 50 mM MOPS buffer (pH 7.0) and dialyzed twice (repeated twice, 30 minutes each time). Complete removal of metal from the prolidase enzymes were evaluated by enzyme assay. To reconstitute the metal center of the enzyme, $CoCl_2$ solution was added to give a final concentration of 75 μM (this corresponds to 3 molar equivalents of Co/prolidase monomer). The samples were then heat treated at 80 °C for 15 minutes and were dialyzed against 1 L deionized 50 mM MOPS buffer (pH 7.0) at 4 °C overnight to remove any

unbound metal. Metal content of the treated protein samples was determined using an ICP emission spectrometer at the North Carolina State University Analytical Service Laboratory.

2.2.6 Circular Dichroism spectroscopy

In order to prevent metal ion contamination arising from the protein purification process, the pure prolidase solution used for CD analysis was prepared using the same method indicated in Metal content analysis, with the exception that 10 mM MOPS, pH 7.0 was used in place of 50 mM MOPS to remove all metal ions and recover the metal center. The protein sample concentration was then adjusted to 10 μM and was analyzed using a J-600 CD instrument (Jasco Ltd, Essex, UK) set for spectral analysis from 200 nm to 320 nm. Sealed cuvettes with a 0.1 cm path length were used in the far-UV region, and sealed cuvettes with a 1.0 cm path length were used in the near-UV region. Photomultiplier high voltage did not exceeded 500 V in the spectral regions measured. Each spectrum was averaged 8 times and all measurements were performed under nitrogen flow.

2.3 RESULTS AND DISCUSSION

2.3.1 Purification of wild type and mutated prolidases

The D209A-, H284A-, H284L-, E313L-, and E327L-prolidase mutants were designed to systematically perturb the cobalt centers (as shown in Figure. 2-1) in order to determine the resulting effect on metal content and enzymatic activity compared to wild type prolidase. Overexpression of the wild type and the five mutant *P. furiosus* prolidases was induced in the T7-RNA polymerase producing *E. coli* strain BL21 (λDE3). The successful induction of the wild type and mutant *P. furiosus* prolidases was confirmed by enzyme activities in cell extracts and in heat-treated crude cell extract (incubation at 80 °C for 30 min), as well as by the appearance of a protein band corresponding to the size of prolidase (42 kDa) on an SDS-polyacrylamide gel (shown in Figure. 2-2). The wild type, D209A-, E327L-, H284A- and H284L- prolidases were each successfully purified by a multi-column chromatography strategy. However, we were unable to purify the E313L-prolidase. The E313L-prolidase protein was highly mis-folded and remained aggregated and recalcitrant to purification techniques. Since the E313L-, H284A- and H284L-prolidases were each designed to affect the Co1 binding site, we did not continue further analyses with the E313L-prolidase.

As expected, mutations targeted to either the Co1 or Co2 metal centers of *P. furiosus* prolidase significantly reduced prolidase activity. As shown in Table 1, the D209A-prolidase, which has a mutation affecting the Co2 metal center, had >1,300-fold reduction in activity compared to wild type prolidase. Single mutations affecting the Co1 metal center (H284A- or H284L-prolidase) had decreases of >2000-fold or 1,900-fold,

respectively, compared to wild type prolidase activity. Mutation of the glutamate-327 residue (E327L-prolidase), which serves as a bidentate ligand for both the Co1 and Co2 metal-binding centers, resulted in no detectable activity. The activity data for the mutant prolidases confirm that occupation of both Co1 and Co2 is essential for prolidase activity, and that occupation of only the Co1 center provides the next highest activity, followed by occupation of only the Co2 binding site.

2.3.2 Physical properties of the mutant P. furiosus prolidases

In an effort to definitively determine which of the Co-binding centers is tight-binding and which is loose-binding, ICP emission spectrometry was used to evaluate the Co content of the purified wild type and mutant *P. furiosus* prolidases. From the Co-content data presented in Table 1, it was seen that D209A-prolidase (Co2 center mutant) contained 0.7 Co per subunit, a value close to that of the wild type enzyme (0.76 Co/subunit), suggesting that D209A-prolidase retains the tightly-bound Co like the wild type prolidase does. H284L-prolidase (Co1 center mutant) contained 0.28 Co/mol, which is 36% of the Co content of the wild type prolidase, indicating that the mutation targeted at the Co1 center disrupts binding of the normally tightly-bound Co. E327L-prolidase (Co1 and Co2 bidentate ligand mutant) contained only 4% of cobalt bound to the wild type (0.03 Co/ per subunit), confirming that disruption of both the Co1 and Co2 metal centers would prevent specific binding of Co to the enzyme. The loss of Co atoms found in the wild type and mutated enzymes is consistent with our expectations that Co^{2+} would be partially removed during the purification process, with the degree of loss depending on the tightness of the Co^{2+} binding sites. These metal content data, therefore, indicated that

aspartate-209 participates in liganding the loose-binding Co (Co2), while the histidine-284 residue is involved in liganding the tight-binding Co (Co1).

The metal analysis data also indicated that the H284A-prolidase contained 1.88 Co/subunit, which is inconsistent with the determination that histidine-284 serves as a ligand for the tight-binding Co center, Co1. It was thought; therefore, that the anomalously high level of Co bound to the H284A-prolidase mutant was the result of non-specific binding of Co to partially mis-folded protein. To determine whether this interpretation was correct, the secondary and tertiary structures of the wild type and all of the mutant *P. furiosus* prolidases were analyzed by circular dichroism in the far UV region (200 nm-250 nm) and near UV region (250 nm-320 nm) (shown in Figure. 2-3).

In the far UV region, D209A-prolidase and H284L-prolidase showed a similar CD spectrum to wild type prolidase, indicating that they contain similar proportions of α-helix and β-sheet in their secondary structures (Figure. 2-3A). In the near UV region, the spectra of both D209A-prolidase and H284L-prolidase were as great as that of wild type prolidase, indicating an equivalent tertiary structure (Figure. 2-3B). A red shift of absorption maximum and increased absorption in the D209A- and H284L-prolidase spectra were also observed, suggesting that some aromatic groups that were normally folded inside the protein structure were now surface accessible because of the mutation. Unlike D209A-prolidase and H284L-prolidase, the protein structure of E327L-prolidase displayed structural changes at both the secondary and tertiary level with a greater proportion of β-sheet in the secondary structure compared to wild type and some unfolded structure at the tertiary level. The loss of globular structure of E327L–prolidase was anticipated since the change of glutamate to leucine at amino acid position 327

affects both the Co1 and Co2 binding sites, and therefore, the whole metal-binding center is disturbed.

CD analysis of the H284A-prolidase mutant clearly indicated that the protein was unfolded. Apparently replacement of the amino acid histidine at position 284 with the small amino acid alanine dramatically changed the protein's folding order, while substitution of histidine with leucine at this position did not. The highly unfolded nature of the H284A-prolidase is undoubtedly responsible for the high levels of non-specifically bound Co that was observed for the H284A-prolidase mutant. Moreover, the CD spectra data indicate that the difference in the specific activities and metal content of wild type prolidase and the D209A-, H284L- and E327L-prolidase mutants is not due to mis-folded protein structure but rather to the changes of binding ability of these mutant prolidases for cobalt. Because the H284A-prolidase mutant was shown to have anomalous metal binding characteristics and secondary and tertiary structure compared to either the wild type or H284L-prolidase proteins based on metal content and CD analysis, the H284A-prolidase mutant was not subjected to further analysis.

2.3.3 Catalytic properties of wild type and mutated prolidases

Since there is a significant difference between the specific activity of wild type and that of mutated prolidases, we used the relative specific activity to investigate their response to the changes in temperature and Co concentration. As shown in Figure. 2-4 and Figure. 2-5, the relative catalytic activities of wild type and D209A-prolidase have virtually identical responses to changes in temperature and Co^{2+} concentration, as both showed a temperature optimum at 100 °C and a similar activity/temperature profile. For both the wild type and D209A-prolidase, maximal activities were observed when the Co

concentration was 0.5 mM; loss of catalytic activities were observed when the [Co^{2+}] was greater than 0.5 mM, with the decrease of enzyme activities by more than 80% at 10 mM Co^{2+}. In contrast, the H284L-prolidase mutant responded to the temperature and [Co^{2+}] changes in a different way. Although the activity of H284L enzyme also peaked at the temperature of 100 °C, it increased rapidly at temperatures greater than 60 °C. Its maximal activity was observed when the Co concentration was 2 mM, which was 4 times higher than the concentration supporting maximal activity for wild type and D209A-prolidase. In addition, the inhibition effect by Co^{2+} at high concentration is less pronounced for H284L-prolidase, with only a 20% decrease in activity at 10 mM Co^{2+}. These data suggest that the tight-binding metal center in the H284L-prolidase mutant has been disrupted, and therefore, requires a significantly higher concentration of Co^{2+} to yield maximal activity. No activity was detected for the E327L-prolidase mutant in the temperature and Co concentration ranges used in these experiments, suggesting that its metal centers were completely disrupted by the mutation.

Previous gene sequence comparisons, biochemical data, and X-ray crystal structure analysis have shown that *P. furiosus* prolidase contains a dinuclear cobalt metal center with different binding affinities in the catalytic center [9, 22, 23]. Furthermore, it has been shown that Mn^{2+}, but not Fe^{2+}, Ni^{2+}, or Zn^{2+} could support activity in place of Co^{2+} [9]. However, recently it has been proposed that *E. coli* and *P. furiosus* MetAPs, which are classed in the same family with prolidases, are actually Fe-containing monometallic hydrolases rather than Co-containing dinuclear hydrolases as previously thought [25, 26]. This conclusion was based on EPR spectra analysis of *E.coli* MetAP loaded with two equivalents of metal and an EXAFS study of the Co^{2+}- and Fe^{2+}-

substituted enzyme [25], which showed that *E. coli* MetAP needs only one Fe^{2+} ion for catalysis.

In light of the recent findings concerning the nature of the metal center in MetAP, it was desirable to evaluate the activity of wild type and mutant *P. furiosus* prolidases substituted with Co^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, or Zn^{2+}. For these studies, the metal-substituted prolidases were prepared under anaerobic conditions to ensure that the 2+ oxidation state was maintained for the metal center-bound ions. Metal content analysis of the EDTA-treated prolidase proteins verified that the treatment completely removed both the Co1 and Co2 bound metal ions prior to the metal reconstitution. Therefore, the reconstitution process enables the replacement of both the Co1 and Co2 sites in the case of wild type prolidase, the Co1 site in D209A-prolidase and the Co2 site in H284L-prolidase. Under both aerobic (Figure. 2-6) and anaerobic (Figure. 2-7) conditions, the wild type and D209A prolidases responded to the different metals in a similar way, with Co^{2+} providing the highest activities and Mn^{2+} providing the second highest activities under aerobic conditions, and Fe^{2+} providing the highest activities and Co^{2+} providing the second highest activities under anaerobic conditions. For the H284L-prolidase, Mn^{2+} supported maximal activity and Co^{2+} supported the second highest activity under aerobic conditions, while Mn^{2+} provided it maximal activity and Fe^{2+} provided it the second highest activity under anaerobic conditions. In contrast to the previously reported data for *P. furiosus* prolidase, which showed no activity for Fe-substituted prolidase when assayed under aerobic conditions, here we show Fe^{2+} supports maximal activity for the wild type and the D209A-prolidase mutant under anaerobic conditions assay conditions. However, the fact that the total activity reported for the D209A-prolidase was 1.9 U/mg

compared to 1,434 U/mg for the wild type enzyme indicates that a single Fe^{2+} ion bound to the intact tight-binding Co1 site is not sufficient for full activity of the enzyme, suggesting that *P. furiosus* prolidase does not function efficiently as a mononuclear Fe-containing metallohydrolase as has been reported for *E. coli* and *P. furiosus* MetAPs [25-27].

The catalytic features of the cobalt center in *P. furiosus* prolidase are almost identical with that of a number of methionyl aminopeptidases (MetAP) and aminopeptidase P (APP) [16], which are also binuclear metallohydrolases with metal centers consisting of five conserved amino residues: aspartate-97, aspartate-108, histidine-172, glutamate-204 and glutamte-235 (*E. coli* MetAP numbering) [27]. Among these metallohydrolases, type 2 human MetAP, *Hs*MetAP2 (pdb 1bn5) [28], *E. coli* MetAP (pdb 1mat, 2mat) [29, 30], and *P. furiosus* MetAP (pdb 1xgs) [31] are similar to *P. furiosus* prolidase and contain a cobalt-active binuclear metal site with different binding affinities as their catalytic center. The Co1 site was determined to be the high-affinity binding site in *E. coli* MetAP using NMR and EXAFS analysis [25]. The dissociation constants, K_d, of Co1 and Co2 were estimated to be 0.3 ± 0.2 μM and 2.5 ± 0.5 mM, respectively [25]. Similarly in *P. furiosus* MetAP, the dissociation constants for Co1 and Co2 were estimated to be 0.05 ± 0.015 μM and 0.35 ± 0.02 mM, respectively [32]. Thus, these published binding affinities for the Co1 and Co2 binding sites from *E. coli* and *P. furiosus* MetAP are consistent with our analyses for *P. furiosus* prolidase.

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Table 2-1.

Specific activity and cobalt content of purified wild type and mutant *P. furiosus* prolidases

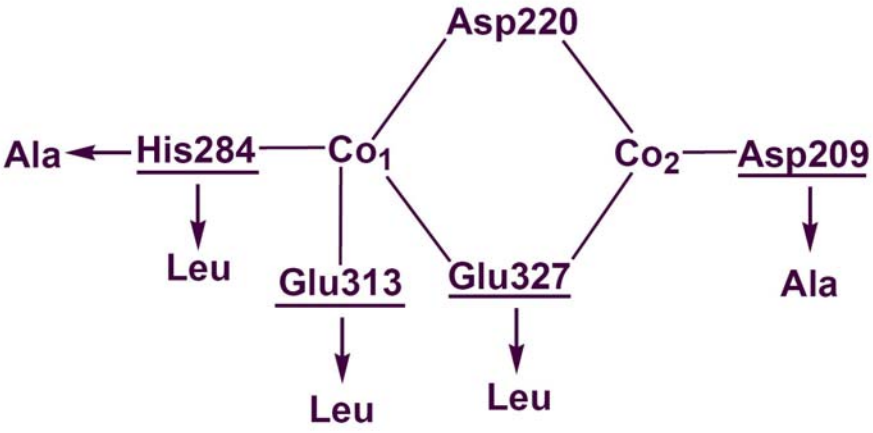
Prolidase	Specific Activity[a] (U/mg)	Cobalt Content (g-atom/subunit)
Wild type	1388 ± 220	0.76
D209A-prolidase (Co2)[b]	1.02 ± 0.21	0.70
H284A-prolidase (Co1)	0.67 ± 0.13	1.88
H284L-prolidase (Co1)	0.73 ± 0.18	0.28
E327L-prolidase (Co1/Co2)	N.D.[c]	0.03

[a]The specific activity is determined for purified protein and is reported as the average ± deviation of 9 values from 3 separate experiments.

[b]The Co-binding center that is disrupted in the mutant is indicated in paranthesis.

[c]N.D., not detected.

(A)



(B)

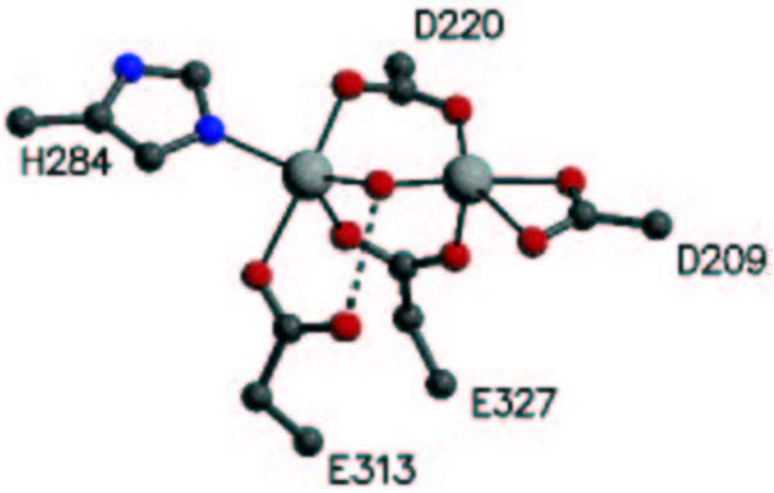


Figure 2-1. Dinuclear metal center active site of *Pyrococcus furiosus* prolidase. The amino acids that were targeted for mutation are shown (A) as is the solved structure of the P. furiosus prolidase active site (B) bound metal is indicated by the gray sphere. The hydrogen bond between glutamate 313 and the bridging hydroxide is shown as a dotted line.

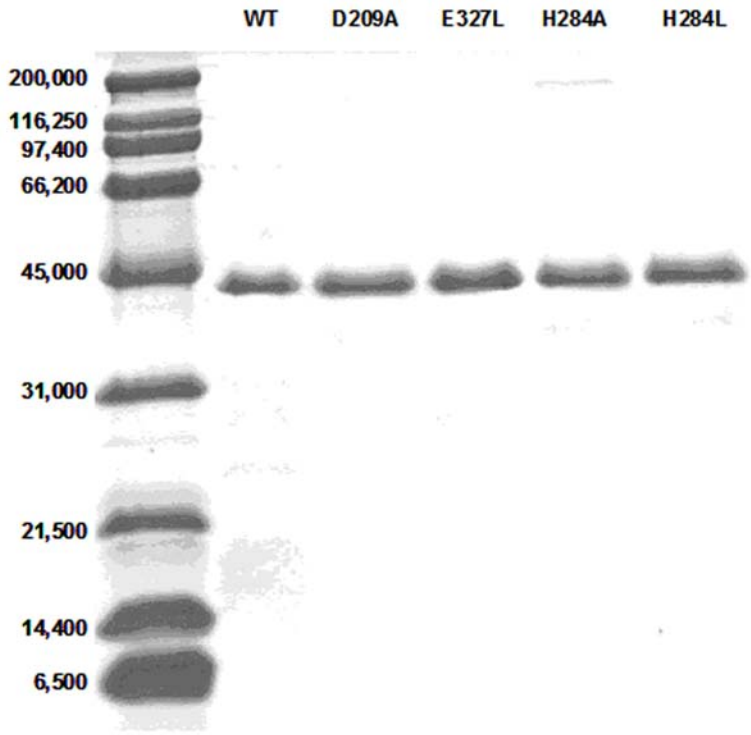
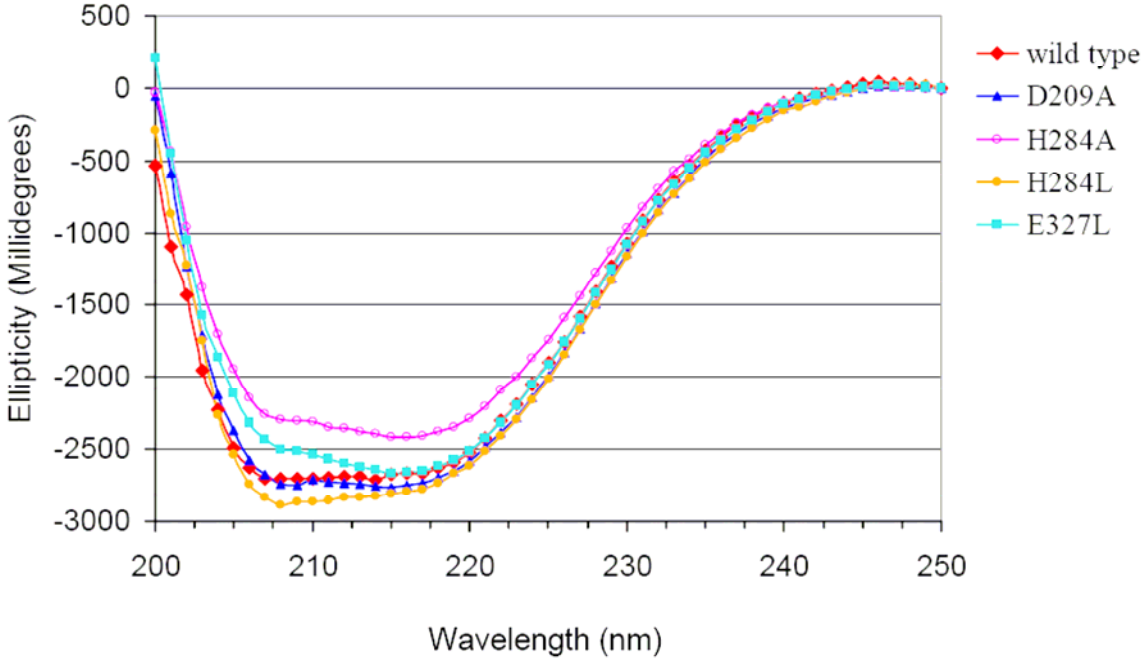


Figure 2-2. SDS-PAGE of purified wild type and mutant *P. furiosus* prolidases (12 % polyacrylamide). Lane 1 contains the molecular weight marker. 5 μg of purified protein was applied to each lane.

(A)



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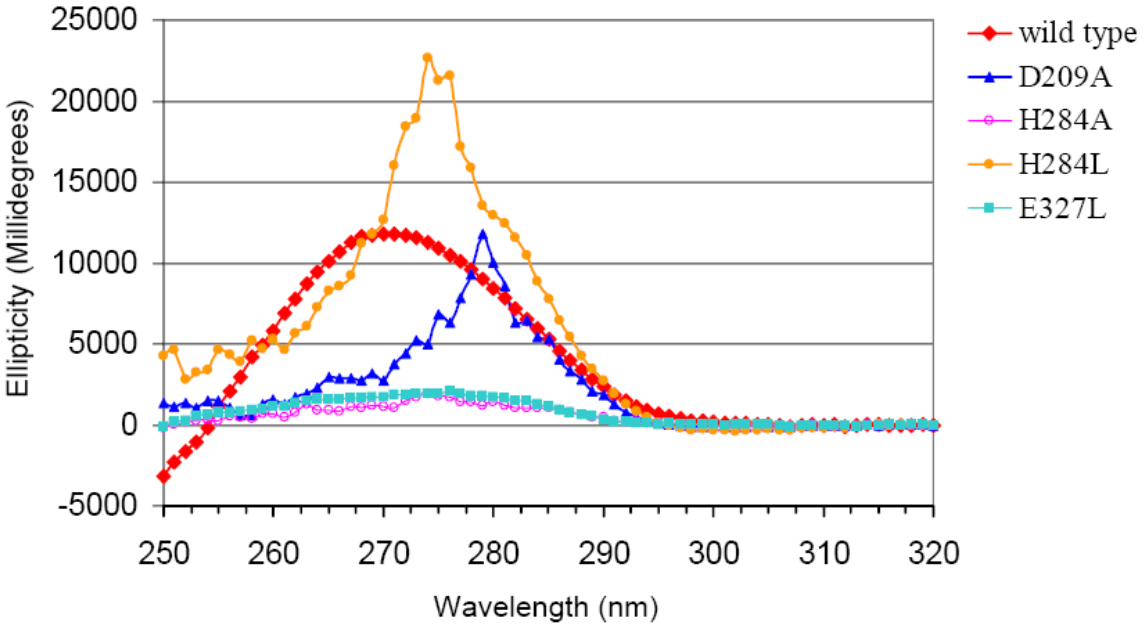

Figure 2-3. CD spectra of wild type and mutated prolidase in the far-UV spectrum region (A) and in the near-UV spectrum region (B).

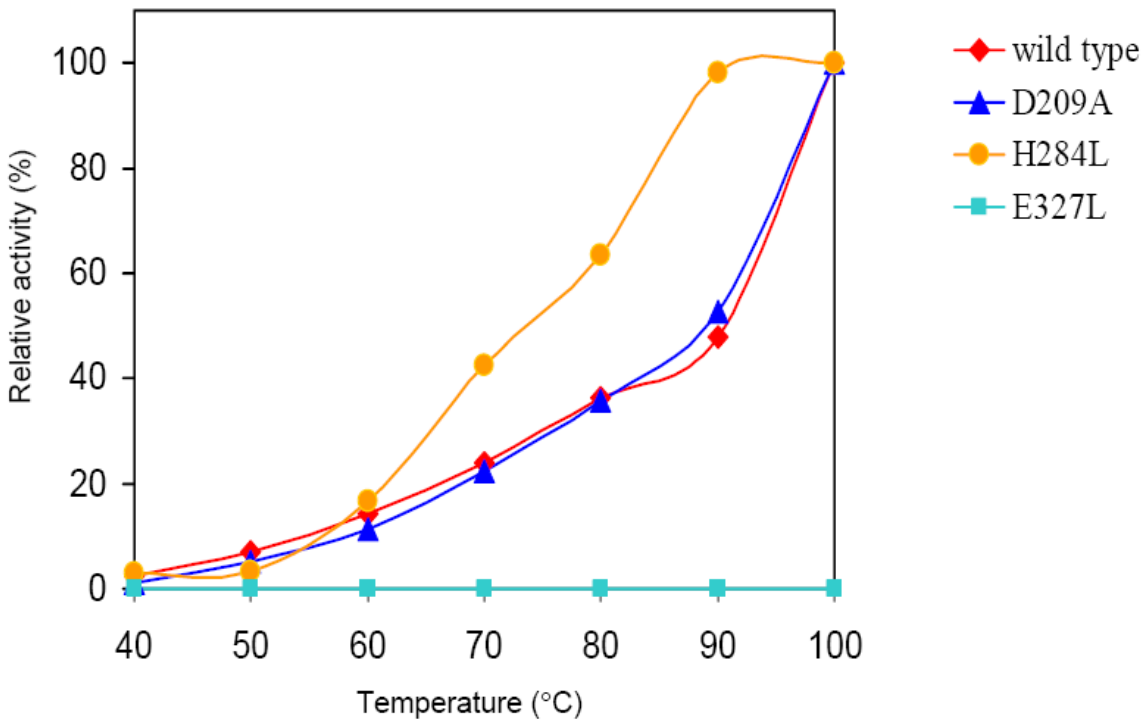

Figure 2-4. Effects of temperature on wild type, D209A-, E327L- and H284L-prolidase activities. 100% specific activity of wild type prolidase is 980 U/mg, 100% specific activity of D209A-prolidase is 0.964 U/mg, and 100% specific activity of H284L-prolidase is 0.90 U/mg.

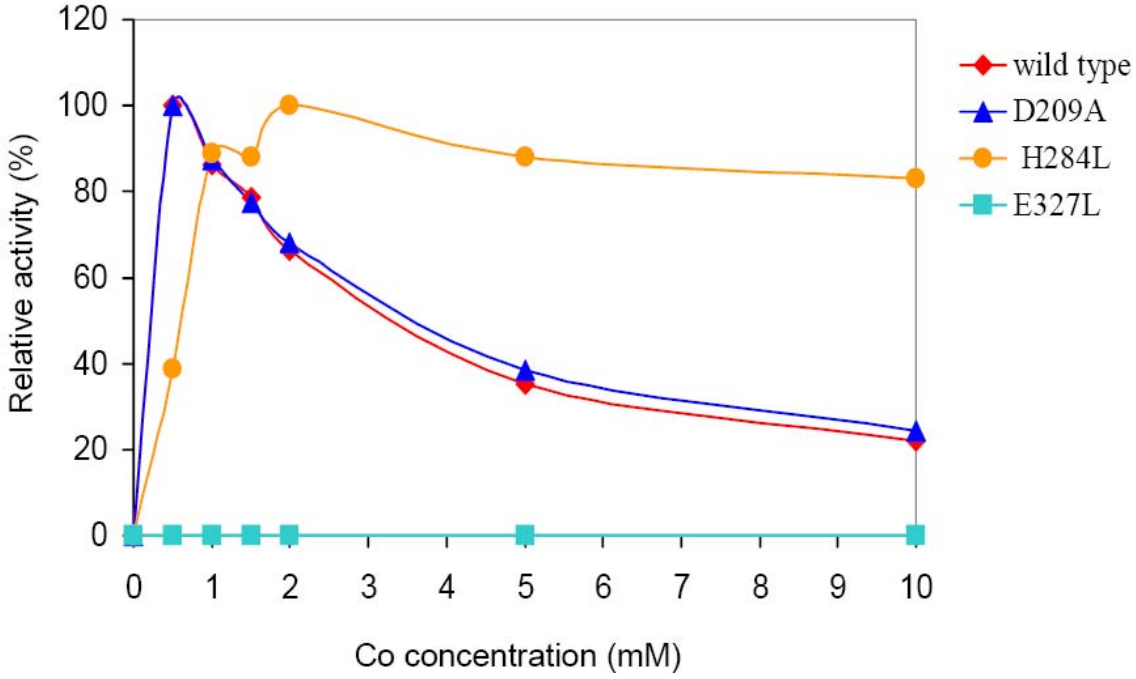

Figure 2-5. Effect of cobalt concentration on wild type, D209A-, E327L-, and H284L-prolidase activities. 100% specific activity of wild type prolidase is 1,375 U/mg, 100% specific activity of D209A-prolidase is 1.42 U/mg, and 100% specific activity of H284L-prolidase is 1.69 U/mg.

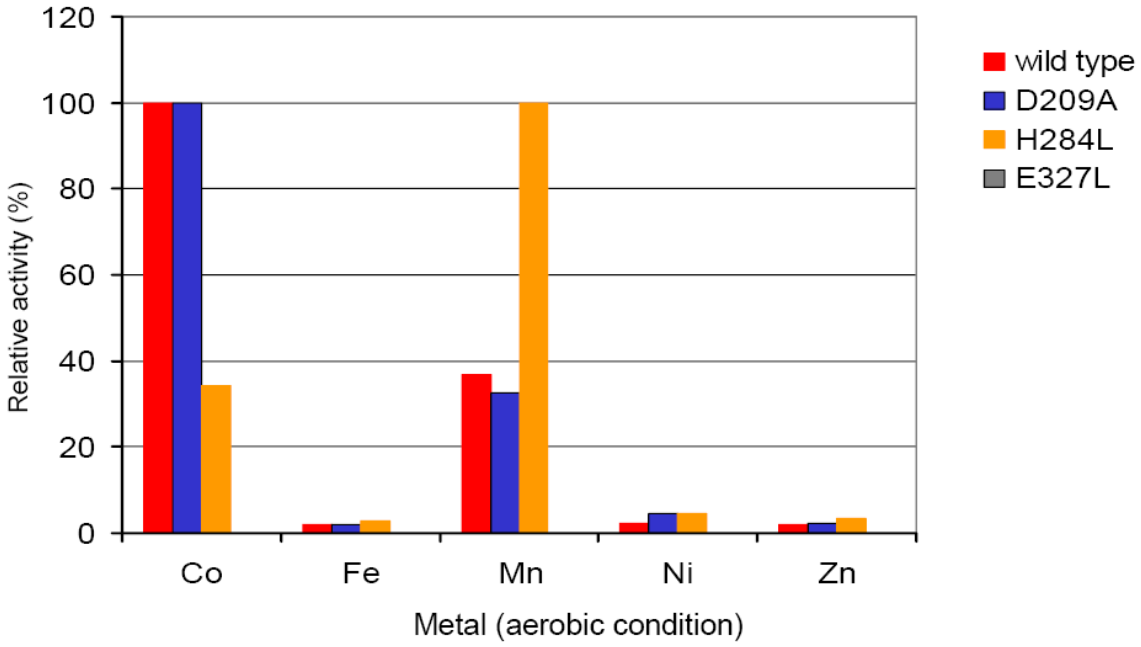

Figure 2-6. Effects of different metals on wild type, D209A-, E327L- and H284L-prolidase activities under aerobic assay conditions. 100% specific activity of wild type prolidase is 1,387 U/mg, 100% specific activity of D209A-prolidase is 1.998 U/mg, and 100% specific activity of H284L-prolidase is 1.81 U/mg. Metal content analyses confirmed removal of Co from both the Co1 and Co2 sites of the metal-stripped prolidases prior to reconstitution with the different metals, and reconstitution of both the Co1 and Co2 sites with the added metals could be confirmed by ICP emission spectroscopy for the wild type prolidase.

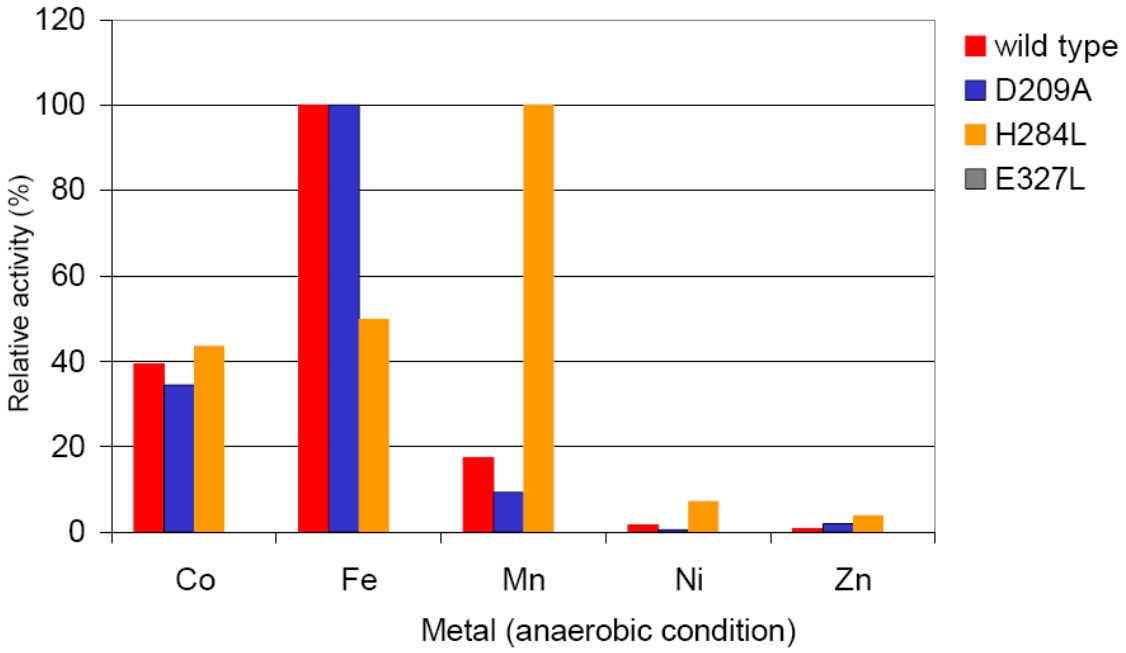

Figure 2-7. Effects of different metals on wild type, D209A-, E327L- and H284L-prolidase activities under anaerobic conditions. 100% specific activity of wild type prolidase is 1,434 U/mg, 100% specific activity of D209A-prolidase is 1.937 U/mg, and 100% specific activity of H284L-prolidase is 1.31 U/mg. Metal content analyses confirmed removal of Co from both the Co1 and Co2 sites of the metal-stripped prolidases prior to reconstitution with the different metals, and reconstitution of both the Co1 and Co2 sites with the added metals could be confirmed by ICP emission spectroscopy for the wild type prolidase.

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CHAPTER 3

Improving the catalytic activity of *Pyrococcus furiosus* prolidase at low temperatures

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ABSTRACT

Prolidase isolated from the hyperthermophilic archaeon *Pyrocossus furiosus* has potential for application under harsh conditions for decontamination and detoxification of organophosphorus compounds contained in certain pesticides and chemical warfare agents. However, this application is greatly restricted by its current limited activity at low temperatures. To obtain a better enzyme for OP nerve agent decontamination and to investigate the structural factors that may influence protein thermostability and thermoactivity, randomly mutated *P. furiosus* prolidase was prepared through PCR mutagenesis, hydroxylamine mutagenesis and mutation-prone XL1-Red mutagenesis. An *Escherichia coli* strain JD1 (λDE3) (auxotrophic for proline [Δ*proA*] and has deletions in *pepQ* and *pepP* dipeptidases with specificity for proline-containing dipeptides) was constructed for screening mutant *P. furiosus* prolidase expression plasmids. When mutated prolidase expression plasmids were transformed into JD1 (λDE3) and were plated on minimal media supplemented with 50 μM Leu-Pro as the only source of proline, only those *E. coli* cells expressing mutant *P. furiosus* prolidase that was active at the room temperature supplied proline to the cells from the hydrolysis of Leu-Pro and formed colonies. By using this positive selection, two *P. furiosus* prolidase mutants with improved activity at low temperatures were isolated. These mutants were further characterized to obtain better understanding of substrate catalysis at both low and high temperature and the relationship of these features with thermoactivity and thermostability.

3.1 INTRODUCTION

Organophosphorus compounds (OPs), one of the most frequently occuring components in pesticides and chemical warfare agents, are highly toxic and often hard to degrade. Examples of these OPs include: the serine protease inhibitor diisopropyl fluorophosphates (DFP), and different G-type nerve agents such as soman (GD; *O*-pinacolyl methylphosphonofluoridate), sarin (GB; *O*-isopropyl methylphosphonofluoridate), and GF (*O*-cyclohexyl methylphosphonofluoridate). Exposure to these OP compounds will irreversibly inhibit peripheral and central acetylcholinesterase (AChE). AChE is responsible for terminating the action of the neurotransmitter acetylcholine. An inhibition of this enzyme results in an accumulation of acetylcholine, which causes an over stimulation of muscarinic and nicotinic receptors, and even produces serious nerve agent poisoning including hypersecretion, convulsions, respiratory distress, coma and death [1].

Current decontamination solutions to remove those toxic OP compounds include the cleaning solution DS2 and bleach [2, 3]. Despite their effective decontamination of OP compounds, both DS2 and bleach are very corrosive and result in hazardous waste. It has been reported that an organophosphorus acid anhydrolase (OPAA) from *Alteromonas* sp., which was later determined to be a prolidase, can hydrolyze those OP compounds by cleaving P-F, P-O, P-CN and P-S bonds [4, 5]. Furthermore, prolidases isolated from other various sources have also been reported to hydrolytically cleave bonds of OP compounds [6-9]. Although the specific catalysis mechanism for these activities is not clear at this stage, it has been noted that there exists fortuitous similarity of the OP compounds in size, shape and surface charges, to X-Pro dipeptides, which are the natural

substrates for prolidase [10-13]. These findings have suggested a potential use of prolidases to decontaminate and detoxify the OPs exposed surfaces without introducing new hazardous waste or destruction of contaminated surfaces.

Prolidase is a ubiquitous dipeptidase that specifically cleaves dipeptides with proline at the C-terminus (NH_2-X-/-Pro-COOH). This enzyme is widespread and has been isolated and characterized from a variety of sources, from mammalian tissues [14-18] to bacterial and archaeal organisms [19-25]. The majority of these prolidases contain a common "pita-bread" fold with a dinuclear metal center, which is generated by five conserved amino acid residues [26]. This structural nature and the substrate specificity prolidases exhibit are quite similar to some other metallopeptidases, such as methionine aminopeptidase (MetAP) and Aminopeptidase P (AAP), and so are classified into the same metallopeptidase subclass [26]. The enzyme activity of prolidases requires divalent cations such as Zn^{2+}, Mn^{2+}, or Co^{2+} [26, 27]. It is unclear why prolidases from different sources utilize different metals in the active site, however the combination of spectroscopic and structural data from APP and MetAP indicate that the metal center plays an essential role in conversion of a water molecule present in the active site to a nucleophilic hydroxide ion and then stabilizing the resulting putative tetrahedral intermediate [26].

The established physiological role of prolidase is to participate, in concert with other endo- and exo-peptidases, in the degradation of intracellular proteins and in proline recycling in cells. In humans, prolidase even mediates the final step of tissue collagen degradation [28, 29]. Prolidase deficiency in humans will cause the accumulation of C-terminal proline-containing dipeptides to toxic concentrations in individuals [17, 29], and

results in a disease characterized by various skin manifestations accompanied by mental retardation, facial dysmorphism and susceptibility to pyogenic infections [28, 29].

Among all prolidases that have been characterized so far, the ones that have been isolated from mesophilic sources are only maximally active at temperatures up to 55°C. However, the prolidase that was isolated from the hyperthermophilic archaeon *Pyrococcus furiosus* has a maximum activity at 100°C. In addition, both native *P. furiosus* prolidase and recombinant *P. furiosus* prolidase produced in *E. coli* possess good thermostability, with the former displaying no activity loss after 12 hours incubation at 100°C and the latter exhibiting 50% activity loss after 6 hours incubation at 100°C [21]. The ability to maintain activity at extreme high temperature makes *P. furiosus* prolidase ideal for OPs decontamination and detoxification, where harsh conditions, such as high temperatures, organic solvents and denaturants, are often required [30]. Importantly, it was recently discovered that *P. furiosus* prolidase indeed can hydrolyze DFP, a model for the G-series OP compounds, with a specific activity of ≈30 units/mg when assayed at 95°C (data obtained from Dr. Joseph DeFrank of the U.S. Army Edgewood Research, Development and Engineering Center). The *P. furiosus* prolidase specific activity compares favorable with activities of prolidases from human and squid, which exhibit specific activities of 10-75 units/mg when assayed at 30°C.

Despite the advantage *P. furiosus* prolidase exhibits under high temperature conditions, the use of *P. furiosus* prolidase for OPs decontamination and detoxification is quite restricted by the fact that this enzyme displayed a narrow range of operational temperature. Like many other enzymes isolated from hyperthermophiles, *P. furiosus* prolidase has only 50% activity at 80°C and displays little activity at temperatures below

50°C [21]. Therefore, it is desirable to attempt to prepare and screen *P. furiosus* prolidase mutants that demonstrate increased activity against OP compounds at low temperature while maintaining its thermostability.

In addition to our goal of identifying a better enzyme for OP compound decontamination, we also set out to investigate the structural factors governing activity and stability of thermophilic enzymes at high temperatures. Thermophilic enzymes generally have negligible activity at low temperature (temperatures < 55 °C). While cold denaturation cannot explain this behavior [31], some recent studies suggest that it might be that their conformational rigidity reduces catalytic activities at low temperature [32-35]. Isolation of low-temperature-adapted *P. furiosus* variants should reveal structural elements that are responsible for its conformational rigidity, and therefore provide insight into this theory. It is also noted that *P. furiosus* is a good model for this study, since the crystal structure and the reaction mechanism of *P. furiosus* have been well studied [3, 26]. For these reasons, we constructed a random-mutated *P. furiosus* prolidase gene library and screened this library for increased activity at room temperature. Two mutants were isolated using the screening procedure and were further purified and characterized, with the aim to obtain insight into substrate catalysis at both low and high temperature and into the relationship of these features with thermoactivity and thermostability.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains, media and materials

The *E. coli* K-12 derivative NK5525 (*proA*::Tn*10*) was used to construct the selective strain JD1(λDE3) for screening of cold-adapted *P. furiosus* prolidase variants. The *P. furiosus* prolidase expression plasmid pET-prol was prepared by cloning the *P. furiosus* prolidase gene into pET-21b vector at the restriction sites *BamH*I and *Not*I [21]. Bacteria were cultured either in Luria-Bertani (LB) broth or M9 selective minimal medium supplemented with 0.2% glucose, 1 mM $MgSO_4$, 0.05% $VitB_1$, 20 μM IPTG, 50 μM Leu-Pro. Ampicillin (100 μg/ml), Kanamycin (50 μg/ml), Chloramphenicol (34 μg/ml) and Tetracycline (6 μg/ml) were added into the medium when required.

3.2.2 Construction of selective strain JD1 (λDE3)

The selective strain JD1 (λDE3) was constructed by the following three steps:

1) Integration of λDE3 prophage into NK5525 chromosome

Integration of λDE3 prophage into the *E. coli* strain NK5525 chromosome was carried out by using a λDE3 lysogenization Kit (Novagen). Briefly, NK5525 cells were cultured in LB supplemented with 0.2% maltose, 10 mM $MgSO_4$ and 6 μg/ml tetracycline at 37°C to an OD_{600} of 0.5. From this, 5 μl of NK5525 cell culture was then mixed with 10^8 pfu of λDE3, 10^8 pfu of helper phage (B10) and 10^8 pfu of selection phage (B482), This host/phage mixture was incubated at 37°C for 20 minutes and was plated on LB agar plates containing tetracycline, which was then incubated at 37°C

overnight. The surviving colonies were further verified to be λDE3 lysogens by their ability to support the growth of the T7 tester phage (4107).

2) Permanent disruption of *proA* in NK5525(λDE3)

proA in NK5525 was originally disrupted by insertion of the tetracycline resistance transposon Tn*10*. To permanently disrupt this gene, Tn*10* was excised by the positive selection on fusaric acid media as described by Maloy and Nunn [36]. Briefly, NK5525(λDE3) cells were grown in LB broth with 6 μg/ml tetracycline until an O.D ≈1.0 was reached. The cell culture was then diluted serially and spread on fusaric acid plates containing fusaric acid for positive selection of tetracycline-sensitive colonies. Colonies growing on the selective plate were spread on fusaric acid plates again for tetracycline-sensitive selection. This process was repeated four times until a stable Tn*10* excision strain was selected. This stain was confirmed as Tc^s and proline auxotrophic by being plated on appropriate agar media.

3) Disruption of *pepP* and *pepQ* in NK5525(λDE3)

Deletion of *pepP* gene and *pepQ* gene was achieved by replacing them with a chloramphenicol resistance cassette and a kanamycin resistance cassette respectively, using the λ-Red system [37]. The chloramphenicol resistance cassette was amplified from the pKD3 plasmid using primer Ec PepP F and primer Ec PepP R (Table 3-1). The 5' terminus of primer Ec PepP F and Ec PepP R comprised 50 and 52 nucleotides identical to the sequences of the 5'-end and 3'-end of *pepP* respectively; the kanamycin resistance cassette was amplified from pKD4 plasmid using primer Ec PepQ F and primer Ec PepQ R (Table 3-1). The 5' terminus of primer Ec PepQ F comprised 51 nucleotides identical to the sequences of *pepQ*, and the 5' terminus of the primer Ec PepQ R comprised 50

nucleotides identical to the downstream sequences of *pepQ*. The PCR amplified products were then purified using a commercial PCR purification kit (Qiagen).

To replace *pepP* with the chloramphenicol resistance gene, NK5525 cells carrying a Red γ-recombinase helper plasmid pKD46 were grown in 10 mL SOB culture with 100 μg/ml ampicillin and 0.02% L-arabinose at 30°C to an OD_{600} of ≈0.6. These cells were then washed three times with ice-cold 10% glycerol and resuspended in 50 μL 10% glycerol. Approximately 50-100 ng PCR product of chloramphenicol resistance cassette was electroporated into the competent cells. Transformants were then selected on LB agar containing 34 μg/ml chloramphenicol and the correct insertion was confirmed by PCR using primer PepP-Up and primer PepP-Dn (Table 3-1). Following the same procedure, *pepQ* was replaced with the kanamycin cassette and was verified by PCR using primer PepQ-Up and primer PepQ-Dn (Table 3-1).

3.2.3 Construction of a pool of pET-prol plasmid carrying randomly mutated P. furiosus prolidase gene

To avoid biased results generated from a single mutagenesis method, random mutations were introduced into the gene encoding *P. furiosus* prolidase by three independent mutagenesis methods: error-prone PCR mutagenesis, hydroxylamine mutagenesis and passage through mutation-prone *E. coli* XL1-red cells.

For PCR mutagenesis, the *P. furiosus* prolidase gene was randomly mutated via error-prone PCR amplification [38] using Taq polymerase (Qiagen) for 30 cycles: (30 s at 95°C, 30 s at 52°C and 90 s at 72°C). Each reaction contained 7 mM $MgSO_4$ and an unequal ratio of dNTPs (0.2 mM dATP and dGTP, 1.0 mM dCTP and dTTP). The DNA

template used for the PCR amplification was pET-prol plasmid and the primers were primer Prol-1 and primer Prol-2 (Table 3-1), with the former containing an engineered *BamH*I site and the latter containing an engineered *Not*I site [21]. The PCR-amplified DNA fragments were digested with *BamH*I and *Not*I, and were religated into pET-21b vectors.

Hydroxylamine mutagenesis was conducted based on a previously described method with slight modification [39]. 7.5 µg of the *P. furiosus* prolidase gene in 50 µl of deionized H_2O was mixed with 40 µl of phosphate-EDTA buffer (0.5 M KPO_4, pH 6.0, 5 mM EDTA) and 80 µl of hydroxylamine-HCl solution, pH 6.0 (0.35 g of NH_2OH-HCl, 0.56 ml of 4 M NaOH, 4.44 ml of distilled H_2O). This mixture was incubated at 37^oC for 18 hours. After the incubation period, the hydroxylamine was removed using the PCR purification Kit (Qiagen). Mutated prolidase gene fragments were digested by *BamH*I and *Not*I, and were religated into pET-21b vectors.

For XL1-red-based mutagenesis, mutation was directed against the intact pET-prol plasmid. Specifically, XL1-red cells (Stratagene) were transformed with pET-prol plasmid DNA. The transformants were then cultured overnight and the plamid DNA was isolated. The overnight culture was then passed into a fresh medium to make a second overnight culture and plasmid DNA was harvested the next morning. After 7 sequential passages, the mutant pET-prol DNA from the last passage was digested using *BamH*I and *Not*I and religated into pET-21b vector.

3.2.4 Screening for increased activity at low temperature

pET-prol plasmids from the mutant *P. furiosus* library were transformed into the selective strain JD1(λDE3), and were plated on M9 selective agar plates. Colonies that grew after being incubated for 3-4 days at room temperature were isolated and grown in 10 mL LB medium at 37 °C with shaking (200 rpm) until an optical density of 0.6-0.8 was reached. IPTG was then added to the cell culture to a final concentration of 1 mM. The induced culture was shaken at 37°C for 3 hours before harvesting the cells.. These cell pellets were lysed using 300 μL of B-per buffer (Pierce Biochemicals) and the resulting cell extracts were used for enzyme activity assays conducted at room temperature (25 °C). Mutant colonies that exhibited at least 3-fold higher activities compared to the cells expressing the wild type *P. furiosus* prolidase were selected and their plasmids were isolated. The prolidase genes present in the isolated plasmids were subsequently sequenced using T7 promoter, prol-3, prol-4 and prol-5 primers (table 3-1) (MWG Biotech, Highpoint, NC).

3.2.5 Generation of G39E-E236V pET-prol plasmid

Production of the G39E-E236V pET-prol plasmid was carried out using a Site-Directed Mutagenessis (Stratagene). In detail, two complimentary oligonucleotide primers (prol G39E-E236V F and prol G39E-E236V R, see table 3-1) containing a mutation from glutamate into valine at amino acid position 236 were designed. These primers were then used in a PCR reaction to amplify G39E pET-prol plasmid templates for 16 cycles (95°C for 30 sec, 55°C for 1 min and 68°C for 7 min). The resulting PCR products were digested with *Dpn*I and transformed into XL1-blue supercompetent cells to

generate the supercoiled form of the G39E-E236V pET-prol plasmid. The correction of this site-directed mutation was verified by DNA sequencing analysis using theT7 promoter, prol-3, prol-4 and prol-5 primers (table 3-1).

3.2.6 Protein purification

Production of *P. furiosus* prolidase and its variants G39E, E236V and G39E-E236V were carried out in *E. coli* BL21(λDE3) that has been transformed with the appropriate pET-prol plasmid. The transformants were grown in 1 L cultures in LB media incubated at 37 °C with shaking (200 RPM) until an optical density of 0.6-0.8 was reached. The expression of mutant prolidase was initiated when IPTG was added to the cell culture to a final concentration of 1 mM. The induced culture was incubated at 37 °C for 3 hours prior to the harvesting of the cells.

To purify G39E-, E236V- and G39E-E236V-prolidase, cell pellets were suspended in 50 mM Tris-HCl, pH 8.0 containing 1 mM benzamidine and 1 mM DTT (each 1 g {wet weight} of cell paste was suspended in 3 ml Tris-HCl buffer). The cell suspension was passed through a French pressure cell (20000 lb/in^2) twice. The lysed cell suspension was centrifuged at 20,000 RPM for 30 minutes to remove any cell debris and the supernatant was heat-treated at 80 °C for 30 min anaerobically. The denatured protein was removed by centrifugation at 20,000 RPM for 30 minutes. $(NH4)_2SO_4$ was slowly added to each heat-treated mutant prolidase extract to a final concentration of 1.5 M, and was then applied to a 20 ml phenyl-sepharose column. The fractions containing the prolidase mutants were further purified by passage through a Q column, followed by a gel-filtration column. For the phenyl-sepharose column chromatography, the binding

buffer was 50 mM Tris-HCl, 1.5 M $(NH_4)_2SO_4$, pH 8.0 and proteins were eluted with a linear gradient of elution buffer: 50 mM Tris-HCl, pH 8.0. For Q column chromatography, the binding buffer used was 50 mM Tris-HCl, pH8.0 and proteins were eluted with a linear gradient of elution buffer: 50 mM Tris-HCl, 1 M NaCl, pH 8.0; For the gel filtration column, the equilibration buffer was 50 mM Tris-HCl, 400 mM NaCl, pH8.0.

3.2.7 Enzyme activity assay

The enzyme activity assay used was based on a previously described method [21, 40] with slight modification indicated below. Briefly, the assay mixture (500 µl) contained 50 mM MOPS buffer (3-[N-morpholino]propanesulfonic acid), pH 7.0, 4 mM Leu-Pro (substrate), 5% (vol/vol) glycerol, 100 µg/ml BSA protein, 200 mM NaCl and 1.2 mM $CoCl_2$, and was incubated at the testing temperature for 5 minutes. The reaction was initiated by addition of the enzyme. The mixture was incubated at the same testing temperature for 10 minutes, and the reaction was stopped by the addition of glacial acetic acid (500 µl) followed by the ninhydrin reagent (500 µl). Color development was achieved by heating the reaction mixture at 100 °C for 10 min. The solution was cooled to 23°C and the absorbance was determined at 515 nm. Specific activities were calculated using the extinction coefficient of 4,570 $M^{-1}\cdot cm^{-1}$ for the ninhydrin-proline complex.

3.2.8 Thermostability measurement

To measure the thermostability of wild type *P. furiosus* prolidase and theG39E-, E236V- and G39E-E236V-prolidases, each enzyme (0.2 mg/ml in 100 mM MOPS, pH

7.0) was incubated in an anaerobic sealed vial at 100°C. Samples, taken at the various time points, were used for enzyme activity assay conducted at 100°C.

3.2.9 Other methods

Protein concentration was determined by the Bradford method with bovine serum albumin as the standard.

3.3 RESULTS AND DISCUSSION

Previous studies have established that both the native and recombinant (produced in *Escherichia coli* using a T7 RNA polymerase-driven expression plasmid pET-21b) forms of *P. furiosus* prolidase exhibit essentially equivalent physical and catalytic properties [21]. Therefore, the recombinant version of the protein, which can be readily expressed and modified, was chosen for use in the study presented here.

3.3.1 Isolation of P. furiosus prolidase mutants with increased activity at low temperature

P. furiosus prolidase has a maximum activity of 1300 U/mg at 100°C and no detectable activity at moderate temperature (temperatures below 40°C) [21]. Therefore, its gene sequence must be altered in order to obtain cold-adapted variants of *P. furiosus* prolidase that exhibit higher catalytic activity at lower temperatures. To achieve this goal, the gene encoding the protein was mutagenized randomly and was ligated back into pET-21b to generate a pool of mutant pET-prol plasmids. Clones of the desired phenotype were positively selected by genetic complementation of an *E. coli* selective strain JD1(λDE3) with deletions of *proA*, *pepP* and *pepQ* genes (Δ*proA*, Δ*pepP*, Δ*pepQ*). *proA* encodes γ-glutamylphosphate reductase (GPR) that participates in the proline synthesis from glutamate in *E. coli*, and *pepP* and *pepQ* are the only two genes in *E. coli* genome encoding dipeptidases with specificity for proline-containing dipeptides. As a consequence of these deletions, JD1(λDE3) is incapable of growing on minimal media supplied with Leu-Pro as the only proline source. Plasmids carrying mutant *P. furiosus* prolidase genes were transformed into JD1(λDE3) cells, and the transformed cells were

plated on minimal media supplied with Leu-Pro and were incubated at room temperature. Since wild type prolidase could not support growth of the host cells at the temperatures that permit growth of *E. coli*, colonies that survived and grew on the selective plates would have to be able to produce cold-adapted variants that had higher activity than the wild type enzyme. Using this positive selection, about 300 colonies were isolated. To avoid false results that came from variation in enzyme production levels, the isolated colonies were further screened for the ones that exhibit at least 3-fold higher activity than the wild type. Results of this study led to the identification of two mutants with increased activity at low temperature, with one containing a single mutation at amino residue 39, a change from glycine to glutamate (G39E) and the other containing a single mutation at amino residue 236 (E236V), a change from glutamate to valine. To verify G39E- and E236V-prolidases indeed exhibit stable increased activity at low temperatures, JD1(λDE3) producing G39E-, E236V- and wild type prolidase respectively were streak on a M9 selective agar plate and were incubated at room temperature for 4 days. As shown in Figure 3-1, G39E- and E236V-prolidases supported the growth of JD1(λDE3) on the M9 selective plate at room temperature. However, wild type prolidase did not.

To avoid bias results that may be generated from single mutagenesis method and to construct a mutant pool with good variety, three mutagenesis methods (error-prone PCR, Hydroxylamine and passage of the prolidase expression plasmid through mutation-prone *E. coli* XL1-red cells) were used in our study to introduce mutations into *P. furiosus* prolidase gene. This strategy proved to be successful: the G39E-prolidase mutant was generated from XL-red cell mutagenesis and the E236V-prolidase mutant was generated from PCR mutagenesis.

3.3.2 Location of amino acid substitution in the mutants with increased activity at low temperature

The three-dimensional model of *P. furiosus* prolidase has been solved and well evaluated [3], and this model was used to analyze the position and possible interactions of each of the amino acid substitutions found in G39E- and E236V-prolidase mutants.

As shown in Figure 3-2, Gly39 is located on a short peptide chain of the N-terminal domain of *P. furiosus* prolidase. This residue has been proposed, in concert with other hydrophobic residues (Pro36, Leu37 and Gly38) in this short peptide chain, to participate in filling the active site in the neighboring subunit and therefore to inhibit the binding of peptides longer than two residues [3]. Furthermore, its adjacent residue Leu37 is located in the substrate binding pocket and directly interacts with the substrate at the P_1 position, together with other hydrophobic residues Ile181, Phe178 and Ile90 in the C-terminal domain of the neighboring subunit. For this reason, the substitution of a hydrophobic residue glycine to a hydrophilic residue glutamate could weakens the hydrophobic environment of the active site and possibly introduces a cavity for the access of substrates.

The second mutant that was isolated, E236V-prolidase, involved a change of a glutamate residue located in a α–helix of the C-terminal domain of *P. furiosus* prolidase. Although it is a protein surface residue and does not directly interact with the active site, this residue and several adjacent residues are highly conserved in prolidases from other organisms (shown in Figure 3-3), which strongly suggests that interactions in this region are critical for correct catalysis. In other words, this region may interact with the active

site indirectly. Therefore, a replacement of the polar residue glutamate with the nonpolar residue valine may cause the disruption of networks of hydrogen bonds, and result in a more flexible structure for the entrance of substrates.

3.3.3 Purification of G39E-, E236V- and G39E-E236V-prolidases

To gain more insight in the effects of cold-adapted substitutions (G39E, E236V) on the *P. furiosus* prolidase, another variant G39E-E236V (a combination of G39E and E236V) was produced. G39E-, E236V-prolidases and the resultant double-mutant enzyme G39E-E236V prolidse were purified from a large-scale expression. Specifically, the G39E-, E236V- and G39E-E236V-prolidases were overexpressed in BL21(λDE3) cells and were purified through a multi-column chromatography strategy. As shown in Figure 3-4, each protein was successfully purified and has a molecular weight of 42 kDa. These protein samples were used for the subsequent enzyme assays.

3.3.4 Effects of the amino acid substitutions on the activity of P. furiosus prolidase at a range of temperatures

To study whether the cold-adapted substitution affects the activity of *P. furiosus* prolidase over a range of temperatures, catalytic activities of G39E-, E236V- and G39E-E236V-prolidase were measured from 10°C to 100°C (shown in Figure 3-5). As expected, both G39E and E236V exhibited increased activity at lower temperatures. Specifically, G39E showed higher activity than the wild type between 30°C and 70°C, with an average increased rate of 1.2 fold and highest increase rate of 1.5 fold at 40°C. Compared to G39E-prolidase, E236V-prolidase has a relatively small range of increased

activity. It displayed increased activity between 10°C and 30°C, with an average increase rate of 1.3 fold and highest increase rate of 1.5 fold at 20°C. Since the G39E mutation is an active-site associated mutation while the E236V substitution is a protein surface associated mutation, differences between G39E and E236V in this assay might indicate that cold-adapted substitution in the active site has a greater effect on enzyme catalysis at different temperatures.

It is intriguing to find that the increased activities of both the G39E and E236V mutations at low temperature came at the cost of decreased activity at high temperature. Specifically, G39E-prolidase has lower activity than wild type above 70°C and has 75% wild type activity at 100°C; E236V-prolidase has lower activity than wild type above 40°C and has only 80% wild type activity at 100°C. This activity decrease is not due to decreased thermostability at high temperature. In fact, it has been previously reported by Lebbink [41] and Suzuki [42] that increased activity at low temperature is accompanied with decreased activity at high temperature in the screen for cold-adapted hyperthermophilic enzymes. Although the exact mechanism behind this is still unclear at this stage, it has been proposed that an improper fitting of the substrate caused by the change of the binding pocket may play an important role [43]. This interpretation agrees well with the results of our study.

A combination of the G39E and E236V mutations, however, does not display an increased rate of activity at low temperature. Instead, its catalytic activity is lower than the wild type at each measured temperature, and only remains 40% of wild type activity at 100°C. The decreased activity of G39E-E236V indicates that the ability for the active

site to catalyze or recognize substrates has been greatly compromised by the double substitutions.

3.3.5 Effect of the substitution on the thermostability of prolidase

To study whether the cold-adapted substitutions affected enzyme thermostability, the G39E-, E236V- and G39E-E236V-prolidases were anaerobically incubated at 100°C, and their catalytic activities were measured at certain time points (see materials and methods). As shown in Figure 3-6, the G39E-, E236V- and G39E-E236V-prolidase mutants retained the original thermostability of wild type protein, each exhibiting a 50% loss of their activities after incubation at 100 °C for 5 hours.

It has been suggested that protein thermostability is not maintained by a single mechanism. Instead, it is fostered by a networks of numerous factors, including increased van der Waals interactions [44], higher core hydrophobicity [45], additional networks of hydrogen bonds [46], enhanced secondary structure propensity [47], ionic interactions [48], increased packing density [49] and decreased length of surface loops [50]. This theory is further supported by our present study. In fact, *P. furiosus* prolidase is able to accommodate single-residue or double-residue substitutions with different properties at either the C-terminal domain or N-terminal domain of *P. furiosus* prolidase, without affecting its thermostability or temperature optimum for catalysis. The observations that thermophilic enzymes have cold-adapted activities without loss of thermostability have also been reported from other studies [41, 42]. In addition, studies of mesophilic enzymes showed that mesophilic enzymes can been engineered to yield better stability without loss

of activity [51, 52]. These results, including ours, support the idea that thermostability and activity can be controlled by separate molecular determinants [43].

3.3.6 Effect of the substitution on the pH optimum of prolidase

Effects of the cold-adapted substitution on protein pH optimum were analyzed by measuring activities of G39E-, E236V- and G39E-E236V-prolidase mutants at 100°C from pH 4 to pH 10 with wild type as the control. As shown in Figure 3-7, the catalytic activities of E236V-prolidase and wild type have virtually identical responses to changes in pH, as both showed optimal activities at pH 5 and a similar activity/pH profile. E236 is located on the surface protein subunit, a substitution of glutamate to valine at this position does not seem to affect prolidase transition state conformation at different pHs. In contrast, G39E- and G39E-E236V-prolidase mutants responded to pH in a different way. Although the activities of G39E- and G39E-E236V-prolidase also peaked at pH 5, the amount is only 50% of that of wild type for G39E and only 40% of that of wild type for G39E-E236V that were obtained from the same conditions. In addition, their activity is somewhat less affected at pH 6 and pH 7, as 80% and 90% of their activities remained. This result strongly suggests that Gly39 plays an important role in maintaining the integrity of active site, and the substitution of this residue with glutamate greatly reduces enzyme activity at optimal pH and high temperature. It was noticed that the optimal pH for the activity of *P. furiosus* prolidase presented in our study is pH 5, which is different from previous report of pH 7 [21]. The reason for the pH optimum shifting is not known, however it might come from the different process of protein purification or treatment.

3.4 CONCLUSIONS

Using the positive selection strategy we designed, we successfully screened two cold-adapted *P. furiosus* prolidase variants, G39E- and E236V-prolidases, which had significant increased activity at low temperatures. Because of the substitutions occurred at different locations in the protein structure, G39E- and E236V-prolidase each exhibit different responses toward the changes in temperature and pH. G39 is an active-site associated residue. The substitution of this residue to glutamate resulted in increased activity below 70°C but decreased activity at optimal pH and temperature for wild type prolidase. E236 is a protein surface associated residue. A substitution of this residue to valine only resulted in increased activity of prolidase below 30°C. Although decreased activity was also observed at optimal pH and temperature, the decrease rate is lower than that of G39E-prolidase. These results led to our assumption that changes in the conformational freedom of active site residues may have larger effects on enzyme catalysis. A further indication that thermophilic enzymes do have structural space to accommodate amino acid residue substitutions without disruption of its thermostability is provided by the thermostability study of G39E-, E236V- and G39E-E236V-prolidases. While these prolidase variants contained amino acid residue substitutions at either the N-terminal domain, or C-terminal domain, or both domains, they exhibited the same stability as that of wild type. Therefore, here we provided experimental evidence to support the idea that using laboratory evolution, thermophilic enzymes can be engineered to yield low temperature activities without loss of their extreme stability.

In conclusion, our efforts toward the improvement of *P. furiosus* prolidase activity at low temperatures resulted in identification of two amino residues critical in

supporting low temperature activity. These results make it promising to screen *P. furiosus* prolidase variants with much greater activity at low temperatures by further producing *P. furiosus* variants with multiple substitutions.

Table 3-1: PCR primers used in this study

Name	Size (nt)	Sequence(5'-3')	Function of PCR product
Ec PepP F	70	GTGAGATATCCCGGCAAGAGTTTCAGCGTCGCCGTCAGGC CCTGGTGGAGTGTAGGCTGGAGCTGCTTCG	For replacement of *pepP* with Cm cassette
Ec PepP R	70	GACGGATAGCGCGCACCGTGGCGGTTAAATTCGTGGTGAA TTTCGCCTTCCATATGAATATCCTCCTTAG	For replacement of *pepP* with Cm cassette
Ec PepQ F	70	AAATCATATAGCTACCTTACAAGAACGGACTCGCGATGCG CTGGCGCGCTTGTAGGCTGGAGCTGCTTCG	For replacement of *pepQ* with Km cassette
Ec PepQ R	70	TCAGCCACTTTCGCCGCCGGAAGCGCCACCCGCAGCAGAA CGAATGCCTGCATATGAATATCCTCCTTAG	For replacement of *pepQ* with Km cassette
PepP-Up	21	GGTTGGGTCAATCACTTCCTG	For PCR verification of pepP deletion
PepP-Dn	21	AGCATGTGACTCTGGCGCAGT	For PCR verification of pepP deletion
PepQ-Up	20	GCCATCTTCCAGCCAGTCAA	For PCR verificationof pepQ deletion
PepQ-Dn	21	TTGCGGTTCAGCCACCACAGC	For PCR verification of pepQ deletion
Prol-1	41	ATAGGATCCGGTGAGGAGGTTGTATGAAAGAAAGACTTGA A	Cloning of prolidase gene into vector
Prol-2	41	ATAGCGGCCGCCTACATTAATCAGAAAGGCTGAAGTTGTT A	Cloing of prolidase gene into vector
Prol-3	18	TGATGACGTGATAAAGGA	For prolidase gene sequencing
Prol-4	18	TGATATGAAAGCAGCTCT	For prolidase gene sequencing
Prol-5	20	GCCCCTCTCTATCCTCTTGT	For prolidase gene sequencing
Prol G39E-E236V F	35	GCTCCCCCAACGAAAAGCAGCGGGTAATTTATGAG (The mutation site of Glu to Val is underlined)	For generation of G39E-E236V pET-prol plasmid
Prol G39E-E236V R	35	CTCATAAATTACCCGCTGCTTTTCGTTGGGGGAGC (The mutation site of Glu to Val is underlined)	For generation of G39E-E236V pET-prol plasmid

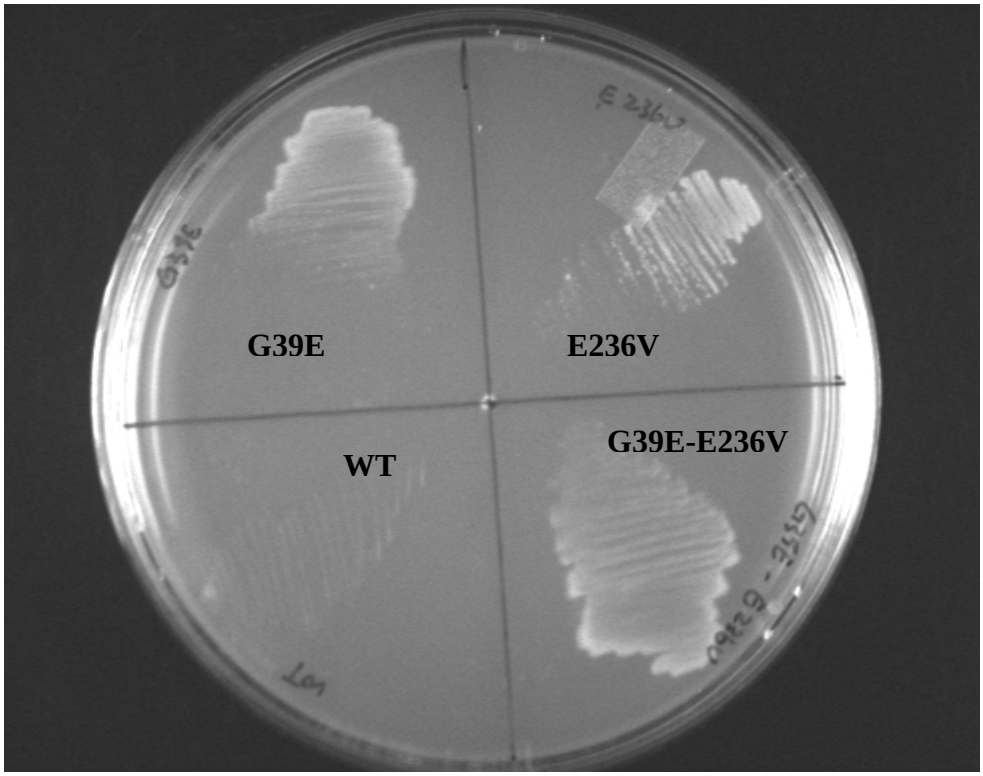


Figure 3-1. Expression of G39E- and E236V-prolidase supports growth of the proline auxotroph *E. coli* strain JD1(λDE3) on the M9 selective agar plate supplemented with leucine-proline. The proline auxotrophic strain JD1(λDE3) that transformed with G39E-, E236V- and wild type pET-prol plasmids, respectively, were streaked on the M9 selective agar plate supplemented with 50 μM Leucine-Proline. This plate was then incubated at room temperature (25°C) for four days. The regions marked with G39E, E236V and WT represent JD1(λDE3) cells transformed with the relative pET-prol plasmids.

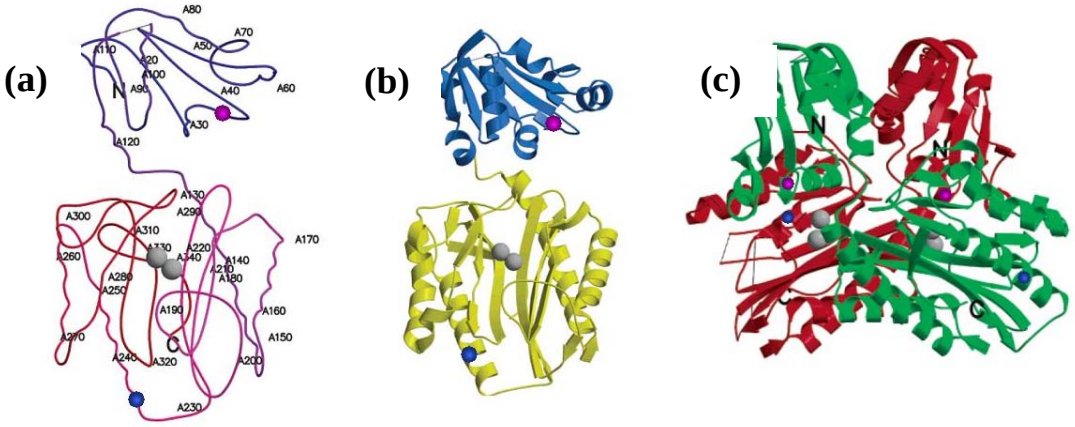


Figure 3-2. Mapping of the mutations in the structure of *P. furiosus* prolidase. G39 is indicated by a purple sphere and E236 is indicated by a blue sphere. (a) Stereoview of the backbone trace of the domain structure; N-terminal domain is in blue and the C-terminal domain is yellow. The metal atoms are gray spheres. (b) Ribbon drawing of the domain structure in the same orientation as (a). (c) Stereoviews of the dimer of prolidase; the subunits A and B are colored green and red, respectively (modified from Maher *et al.*, 2004)

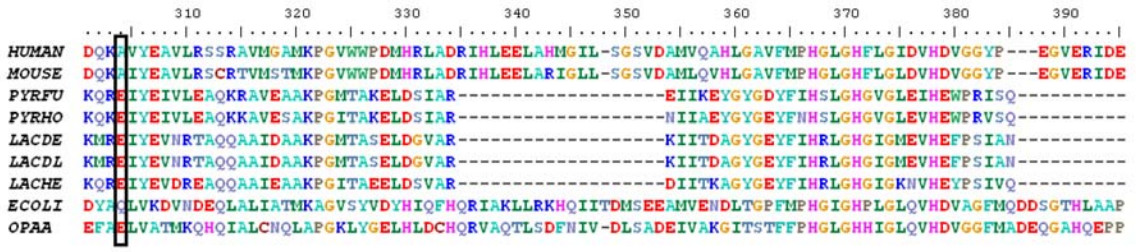


Figure 3-3. Alignment of partial prolidase sequences. Abbreviations are as follows: HUMAN, *homo sapiens*; MOUSE, *Mus Musculus*; PYRFU, *Pyrococcus furiosus*; PYRHO, *Pyrococcus horikoshii*; LACDE, *Lactobacillus delbrueckii subsp. bulgarious*; LACDL, *Lactobacillus delbrueckii subsp. lactis*; LACHE, *Lactobacillus helveticus*; ECOLI, *Escherichia coli*; OPAA, Organophosphorus acid anhydrolase-2, *Alteromonas sp*. The amino acid residue E236V of *P. furiosus* and corresponding residues in other organisms are framed black.

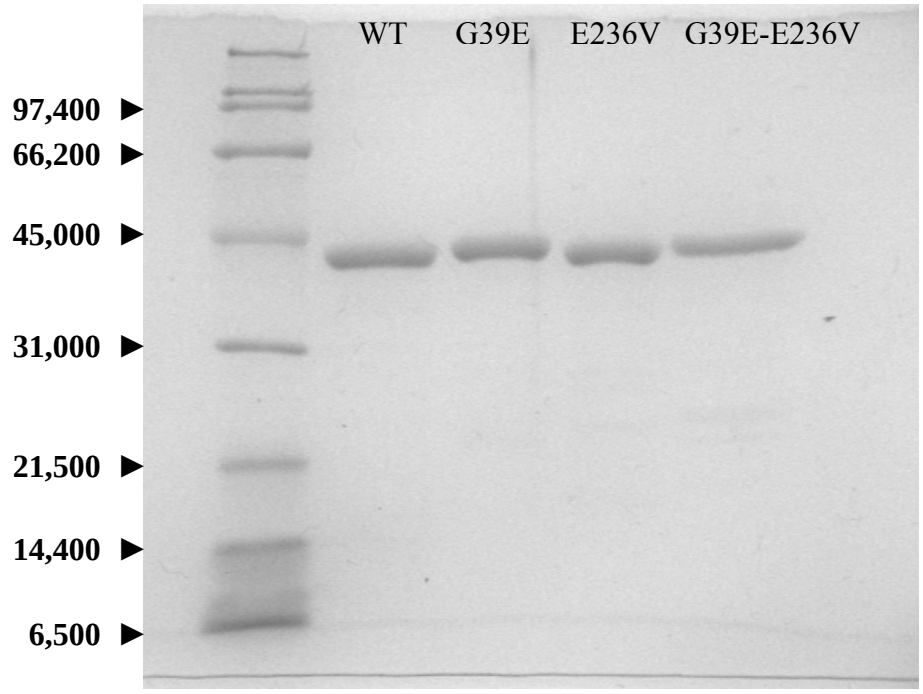
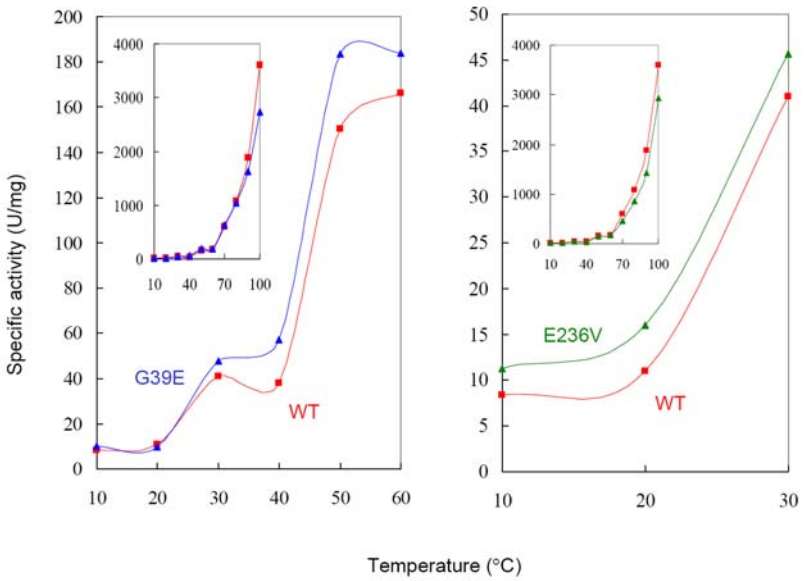


Figure 3-4. Purified wild type, G39E-, E236V- and G39E-E236V-prolidases. Proteins were loaded on an SDS-PAGE gel (12.5% polyacrylamide). Lane 1 contains the molecular weight marker. 3 μg of purified protein was applied to lanes 2-5.



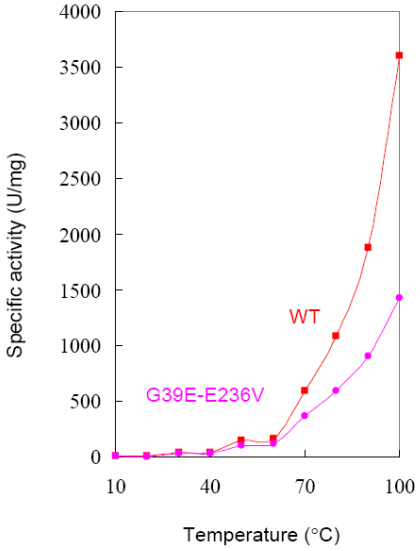


Figure 3-5. Effect of substitution of G39E and E326V on the activity of *P. furiosus* prolidase at different temperatures.

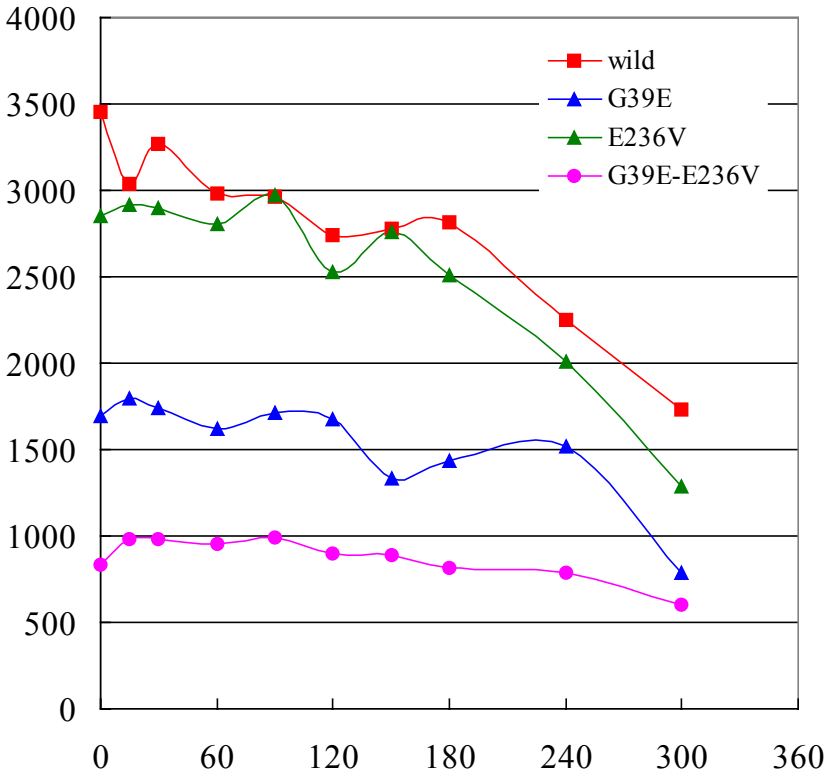


Figure 3-6. Thermostability of wild type, G39E, E236V and G39E-E236V prolidases. Protein samples (0.2 mg/ml) were sealed in anaerobic vials and were incubated at 100°C. Activities were then measured at certain time points until 50% losses were observed.

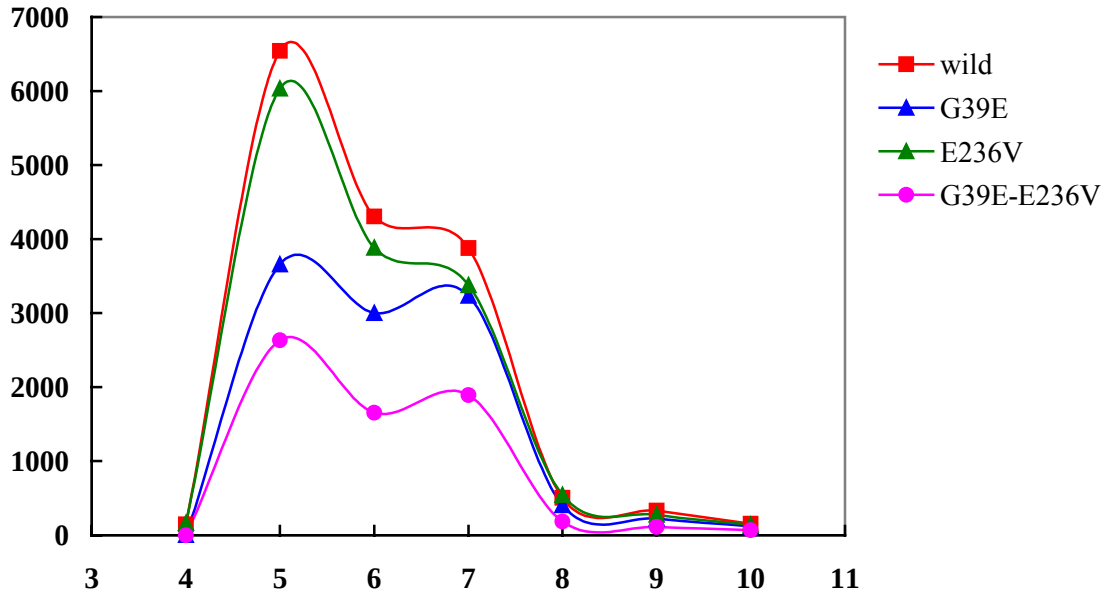


Figure 3-7. Effect of pH on the activities of wild type, G39E-, E236V- and G39E-E236V-prolidase. The following buffers (50 mM each) were used in this study: sodium acetate, pH 4.0 and pH 5.0; bis-Tris-HCl, pH 6.0; MOPs, pH 7.0; EPPS, pH 8.0; CHES (2-[N-cyclohexylamino]-ethanesulfonic acid, pH 9.0; and CAPS, pH 10.0. The assay was carried out at 100°C.

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SUMMARY

The research presented in this dissertation is focused on the understanding and modification of *Pyrococcus furiosus* prolidase for improved application in decontamination and detoxification of organophosphorus compounds.

Previous study suggested this enzyme contained two cobalt atoms with different affinity at the catalytic center for full activation. However no previous study has identified which Co site is tight-binding and which is loose-binding. Chapter 2 presented in this dissertation clearly addressed this question. By using site-directed mutagenesis to modify amino acid residues that participate in binding the Co1 site (E313 and H284), the Co2 site (D209) or bidentate ligand site (E327), metal-content, enzyme activity and CD-spectra analyses of D209A-, H284L- and E327L-prolidase mutants show that *P. furiosus* prolidase contains a dinuclear metal center with different binding affinity, with Co1 serving as the tight-binding and Co2 as the loose-binding metal center. This study not only provides insight into the mechanism of *P. furiosus* prolidase catalytic reaction, but also facilitates designing a better *P. furiosus* prolidase enzyme for industrial application.

Chapter 3 focused on improving the activity of *P. furiosus* prolidase at low temperatures. *P. furiosus* prolidases were randomly mutated using laboratory evolution, followed by a screening at room temperature for increased activity. This study resulted in the isolation of two prolidase mutants with increased activity at low temperatures. Further characterization of these two mutants revealed the relationship of the structural elements of *P. furiosus* with its thermoactivity and thermostability. This study provided

experimental evidence to develop a better prolidase enzyme for OP nerve agent hydrolyzation.

In conclusion, the research presented in this dissertation greatly expanded the current knowledge about *P. furiosus* prolidase structure and function, and provided experimental evidence for the possibility of improving the catalytic activity of *P. furiosus* prolidase at low temperatures using laboratory evolution. This data may guide further isolation of *P. furiosus* prolidase variants with much greater activity at low temperatures by producing mutants with multiple substitutions.